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(54) Title: METHODS AND COMPOUNDS FOR PREVENTION OF GRAFT REJECTION

**(57) Abstract**

Disclosed is a method of preventing graft rejection by inducing a state of local immunosuppression at the transplant site with expression of recombinant proteins by the allograft. Also disclosed is a protein suppressor factor that is secreted by cloned anergic T-cells, blocks interleukin 2 (IL-2) stimulated T-cell proliferation, has an apparent molecular weight of between 10 and 30 kilodaltons, can be inactivated by heating to 65 °C for 15 minutes, blocks interleukin 4 (IL-4) stimulated T-cell proliferation *in vitro*, is non-cytotoxic to T-cells, and does not inhibit the production of IL-2 by T-cells *in vitro*.

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# METHODS AND COMPOUNDS FOR PREVENTION OF GRAFT REJECTION

## Statement as to Federally Sponsored Research

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## Field of the Invention

This invention relates to organ transplantation and to compounds useful in inhibiting rejection of transplanted organs.

## Background of the Invention

For many life-threatening diseases, organ transplantation is considered a standard treatment and, in many cases, the only alternative to certain death. The immune response to foreign cell surface antigens on the graft, encoded by the major histocompatibility complex (MHC) and present on all cells, generally precludes successful transplantation of tissues and organs unless the transplant tissues come from a compatible donor or the normal immune response is suppressed. The best compatibility and thus, longterm rates of engraftment, are achieved using MHC identical sibling donors or MHC identical unrelated cadaver donors (Strom, 1989, In: Organ Transplantation: Current Clinical and Immunological Concepts: 4:8-19; Strom, 1990, Clinical Aspects of Autoimmunity 4:8-19).

The host response to an organ allograft involves a complex series of cellular interactions among T and B lymphocytes as well as macrophages or dendritic cells that recognize and are activated by a foreign antigen (Strom, 1989, In: Organ Transplantation: Current Clinical and Immunological Concepts: 4:8-19; Strom, 1990, Clinical Aspects of Autoimmunity 4:8-19). Co-stimulatory

Clinical Aspects of Autoimmunity 4:8-19). Co-stimulatory factors, primarily cytokines, and specific cell-cell interactions, provided by activated accessory cells such as macrophages or dendritic cells are essential for T cell proliferation. These macrophages and dendritic cells either directly adhere to T cells through specific adhesion proteins or secrete cytokines that stimulate T cells, such as IL-1 and IL-6 (Strom, 1989, In: Organ Transplantation: Current Clinical and Immunological Concepts: 4:8-19; Strom, 1990, Clinical Aspects of Autoimmunity 4:8-19).

IL-1 induces expression of the IL-6 gene in accessory cells. Accessory cell-derived co-stimulatory signals stimulate activation of interleukin-2 (IL-2) gene transcription and expression of high affinity IL-2 receptors in T cells (Pankewycz, et al., 1989 Transplantation 47:318; Cantrell, et al., Science 224:1312; Williams, et al., 1984 J. Immunol. 132:2330-2337). IL-2, a 15 kD protein, is secreted by T lymphocytes upon antigen stimulation and is required for normal immune responsiveness. IL-2 stimulates lymphoid cells to proliferate and differentiate by binding to IL-2 specific cell surface receptors (IL-2R). Expression of the receptor is constitutive in natural killer (NK) cells, but dependent on antigen stimulation in most T and B cells (Kaplan, G., et al., 1992, *Biotechnology* 10:157). IL-2 also initiates helper cell activation of cytotoxic T cells and stimulates secretion of γ-interferon (γ-IFN) which in turn activates cytodestructive properties of macrophages (Farrar, et al., 1981 J. Immunol. 126: 1120-1125). Furthermore, γ-IFN and IL-4 are also important activators of MHC class II expression in the transplanted organ, thereby further expanding the rejection cascade by actually making the grafted organ more immunogenic

(Pober, et al., 1983, J. Exp. Med. 157: 1339; Kelley, et al., 1984 J. Immunol. 132, 240-245).

Similar mechanisms are involved in the development of autoimmune disease, such as type I diabetes. In humans and non-obese diabetic mice (NOD), insulin-dependent diabetes mellitus (IDDM) results from a spontaneous T-cell dependent autoimmune destruction of insulin-producing pancreatic $\beta$ cells that intensifies with age. The process is preceded by infiltration of the islets with mononuclear cells (insulitis), primarily composed of T lymphocytes (Bottazzo, G.F., et al., 1985, *N.Engl. J. Med.* 113:353; Miyazaki, A., et al., 1985, *Clin. Exp. Immunol.* 60:622). A delicate balance between autoaggressive T-cells and suppressor-type immune phenomena determine whether expression of autoimmunity is limited to insulitis or progresses to IDDM. In NOD mice, a model of human IDDM, therapeutic strategies that target T-cells have been successful in preventing IDDM (Makino, et al., 1980, *Exp. Anim.* 29:1). These include neonatal thymectomy, administration of cyclosporine A, and infusion of anti-pan T-cell, anti-CD4, or anti-CD25 (IL-2R) mAbs (Tarui, et al., 1986, *Insulitis and Type I Diabetes. Lessons from the NOD Mouse*. Academic Press, Tokyo, p. 143).

The aim of all rejection prevention and reversal strategies is to suppress the patient's immune reactivity to the graft, with a minimum of morbidity and mortality. Existing immunosuppressive therapies include administration of immunosuppressive compounds such as cyclosporine A, FK506 and rapamycin (First, 1992 Transplantation 53:1-11). Because these agents inhibit proliferation of T cells generally, systemic treatment of patients leads to systemic immunosuppression which carries with it potential complications, including increase risk of infections and cancer (Wilkinson, et

al., Transplant 47:293-296; Penn, 1991 Transplant Proc. 23:1101; Beveridge, et al., 1984 Lancet 1:788). In addition, these immunosuppressive agents cause considerable side effects, including nephrotoxicity, hepatotoxicity, hypertension, hirsutism, and neurotoxicity (Strom, 1989, In: Organ Transplantation: Current Clinical and Immunological Concepts: 4:8-19; Strom, 1990, Clinical Aspects of Autoimmunity 4:8-19; Tilney, et al., 1991 Ann Surg. 214:42-49; Myers, et al., 1984 N. Engl. J. Med. 311:699).

Administration of monoclonal antibodies against the T cell-specific antigen CD3 has been shown to block acute allograft rejection (Mackie, et al., 1990 Transplantation 49:1150). Antibodies directed against the IL-2 receptor, T cell receptor, CD4, and certain cell adhesion molecules such as ICAM-1 have been used (Cosimi, et al., 1990 Surgery 108:406; Cosimi, et al., 1990 J. Immunol. 144:4604; Strom et al., 1989 Kidney Int. 35:1026; Walz, 1990 Transplantation 49:198-201). Anti-IL-2 receptor antibodies have been shown to bring about improved patient and graft survival (Soulillou, et al., 1990 N. Engl. J. Med. 322:1175).

Two novel cytokines, TGF-$\beta$ and IL-10, have recently been identified. These proteins have immunosuppressive activity, and apparently act via different mechanisms on the immune system (Moore, et al., 1990 Science 248: 1230-1234; Vieira, et al., 1991 Proc. Natl. Acad. Sci USA 88:1172-1176; Barnard, et al., 1990 Biochim Biophys. Acta 1032:79-87; Derynck, et al., 1985 Nature 316:701-705).

TGF-$\beta$ exhibits potent immunosuppressive effects including inhibition of B and T cell activation and differentiation and deactivation of activated macr phages (Barnard, et al., 1990 Biochim Biophys. Acta 1032:79-87; Roberts et al., 1990 Handbook of Experimental

Pharmacology 95:419-472). In particular, TGF-$\beta$ inhibits expression of immunoglobulin genes in B cells, decreases IL-2 induced T cell proliferation and differentiation of cytotoxic T cells, and inhibits MHC class II antigen expression on a number of cell types (Kehrl, et al., 1986 J. Exp. Med. 163:1037-1050; Ruegemer, et al., 1990 J. Immunol. 144:1767-1776; Cross, et al., 1990 J. Immunol. 144:432-439; Nelson, et al., 1991 J. Immunol. 146: 1849-1857; Lee, et al., 1987 J. Exp. Med. 166:1290-1299; Wright, et al., 1986 Diabetes 353:1174-1177). TGF-$\beta$ has been shown to inhibit pancreatic islet allograft rejection in mice, suggesting its potential use as an immunosuppressive agent. TGF-$\beta$ is produced by many different cell types, including macrophages, B and T cells, lung and mesenchymal cells, skin cells, platelets, and bone cells (Barnard, et al., 1990 Biochim Biophys. Acta 1032:79-87; Roberts et al., 1990 Handbook of Experimental Pharmacology 95:419-472). In addition, some cancer patients have been observed to show signs of immunosuppression due to secretion of TGF-$\beta$ by the cancer cells (Barnard, et al., 1990 Biochim Biophys. Acta 1032:79-87; Roberts et al., 1990 Handbook of Experimental Pharmacology 95:419-472; Siepl, et al., 1988 Eur. J. Immunol. 18:593-600).

IL-10 was first identified as a product of activated $T_H2$ T helper cells with the ability to inhibit macrophage-dependent cytokine synthesis in $T_H1$ T helper cells (Fiorentino, et al., 1989 J. Exp. Med. 170:2081). IL-10 appears to be expressed by several different hematopoietic cell types, including activated $T_H2$ cells, activated macrophages, mast cells, and B cells (Moore, et al., 1990 Science 248:1230-1234; O'Barra, et al., 1990 Int. Immunol. 2:821; De Waal Malefyt, et al., 1991 J. Exp. Med. 174:1209-1220). IL-10 appears to inhibit the expression of a number of cytokines in macrophages,

including interleukin-6 (IL-6), interleukin-1 (IL-1), interleukin-8 (IL-8), tumor necrosis factor-α (TNF-α), granulocyte/macrophage colony stimulatory factor (GM-CSF) and granulocyte colony stimulatory factor (G-CSF). IL-10 diminishes the antigen-presenting capacity of macrophages via downregulation of MHC class II gene expression on macrophages, and induces expression of MHC class II genes, but not class I genes in unstimulated splenic B cells (Go, et al., 1990 J. Exp. Med. 172:1625-1631; De Waal Malefyt, et al., 1991 J. Exp. Med. 174:915-924).

## Summary of the Invention

In one aspect, the invention features a substantially pure suppressor factor protein or biologically active analog or fragment thereof. The protein is characterized in that it is secreted by cloned anergic T-cells (e.g., IS-2.15 cells), it blocks interleukin 2 (IL-2)-stimulated T-cell proliferation, it has an apparent molecular weight of between 10 and 30 kilodaltons, it can be inactivated by heating to 65°C for 15 minutes, it blocks interleukin 4 (IL-4)-stimulated T-cell proliferation *in vitro,* it is non-cytotoxic to T-cells, and it does not inhibit the production of IL-2 by T-cells *in vitro*.

"Anergic T-cells", as used herein, refers to T-cells that are hyporesponsive or unresponsive to an antigen or mitogen. By "apparent molecular weight" is meant molecular weight as determined by filtration through microconcentrator tubes with multiple membrane size cut-offs, followed by testing of the resulting concentrates and filtrates for T-cell suppressor activity as described in the detailed description to follow.

The invention also features a purified nucleic acid encoding the new suppressor factor of the invention.

The nucleic acid of the invention can be used to alter the effect of IL-2 on cells in a mammal that express, constitutively or transiently, the IL-2 receptor, in order to inhibit IL-2 induced cell proliferation, the proliferation of autoreactive T-cells. The method involves bringing into close proximity of the cell a second cell which is transfected with the nucleic acid encoding the suppressor factor, so that the second cell secretes a protein which causes an alteration of the IL-2 effect. The mammal is preferably a human, and the cell is preferably a T-cell, an endothelial cell lining a blood vessel, or an epithelial cell, most preferably an epithelial cell of the kidney proximal tubule, or an epithelial cell of the gut.

The nucleic acid of the invention can also be used to alter the effect of IL-2 on cells in a mammal that express, constitutively or transiently, the IL-2 receptor. The method involves transfecting the cell with the nucleic acid so that the cell secretes the suppressor factor, causing alteration of the IL-2 effect.

In a related aspect, the invention features a method of altering the effect of IL-4 on cells in a mammal that express, constitutively or transiently, the IL-4 receptor, in order to inhibit IL-4 induced cell proliferation. The method involves bringing into close proximity of the cell a second cell which is transfected with the nucleic acid encoding the suppressor factor, so that the second cell secretes the factor causing an alteration of the IL-4 effect. The mammal is preferably a human, and the cell is preferably a T-cell, an endothelial cell lining a blood vessel, or an epithelial cell, most preferably an epithelial cell of the kidney proximal tubule, or an epithelial cell of the gut.

In a another aspect, the nucleic acid of the invention can be used in a method of altering the effect

of IL-4 on cells in a mammal that express, constitutively or transiently, the IL-4 receptor. The method involves transfecting the cell with the nucleic acid encoding the suppressor factor so that the cell secretes the factor causing alteration of the IL-4 effect.

The suppressor factor protein of the invention can be obtained from any suitable naturally occurring source and can also be made recombinantly. Also included in the invention are biologically active fragments and analogs of the suppressor factor. The term "fragment", as applied to a polypeptide, will ordinarily be at least about 10 contiguous amino acids, more typically at least about 20 contiguous amino acids, usually at least about 30 contiguous amino acids, preferably at least about 40 contiguous amino acids, more preferably at least about 50 contiguous amino acids, and most preferably at least about 60 to 80 or more contiguous amino acids in length. Biologically active fragments of the suppressor factor can be generated by methods known to those skilled in the art.

A suppressor factor polypeptide, fragment, or analog is biologically active if it exhibits a biological activity of naturally occurring IS-2.15 suppressor factor, e.g., the ability to block IL-2 stimulated T-cell proliferation. The ability of a candidate analog or fragment to block IL-2 stimulated T-cell proliferation can be assessed by methods known to those skilled in the art, e.g., by methods described below.

The invention also includes biologically active analogs of the suppressor factor protein of the invention. Analogs can differ from naturally occurring IS-2.15 suppressor factor by amino acid sequence differences or by modifications that do not affect sequence, or by both. Modifications include *in vivo* or *in vitro* chemical derivatization of polypeptides, e.g.,

acetylation, or carboxylation. Also included are modifications of glycosylation, e.g., those made by modifying the glycosylation patterns of a polypeptide during its synthesis and processing or in further processing steps, e.g., by exposing the polypeptide to enzymes that affect glycosylation derived from cells that normally provide such processing, e.g., mammalian glycosylation enzymes. Also embraced are versions of the same primary amino acid sequence that have phosphorylated amino acid residues, e.g., phosphotyrosine, phosphoserine, or phosphothreonine. Analogs can differ from naturally occurring IS-2.15 suppressor factor by alterations of their primary sequence. These include genetic variants, both natural and induced. Induced mutants may be derived by various techniques, including random mutagenesis of the encoding nucleic acids using irradiation or exposure to ethanemethylsulfate (EMS), or may incorporate changes produced by site-specific mutagenesis or other techniques of molecular biology. See, Sambrook, Fritsch and Maniatis (1989), Molecular Cloning: A Laboratory Manual (2d ed.), CSH Press, hereby incorporated by reference. Also included are analogs that include residues other than naturally occurring L-amino acids, e.g., D-amino acids or non-naturally occurring or synthetic amino acids, e.g., $\beta$ or $\gamma$ amino acids. Analogs of the invention, to be biologically active, will generally exhibit at least 70%, more preferably 80%, more preferably 90%, and most preferably 95% or 99%, homology with all or part of a naturally occurring IS-2.15 suppressor factor amino acid sequence. The length of comparison sequences will generally be at least about 8 amino acid residues, usually at least 20 amino acid residues, more usually at least 24 amino acid residues, typically at least 28 amino acid residues, and preferably more than 35 amino acid residues.

"Homologous", or "homology", as used herein, refers to the subunit sequence similarity between two polymeric molecules, e.g., between two nucleic acid molecules, e.g., two DNA molecules, or two polypeptide molecules. When a subunit position in both of the two molecules is occupied by the same monomeric subunit then they are homologous at that position. The homology between two sequences is a direct function of the number of matching or homologous positions, e.g., if half, e.g., 5 of 10, of the positions in two compound sequences are homologous then the two sequences are 50% homologous, if 90% of the positions, e.g., 9 of 10, are matched or homologous the two sequences share 90% homology. By way of example, the DNA sequences 3'ATTGCC'5 and 3'TATGGC'5 are 50% homologous.

The invention also includes proteins encoded by DNA that hybridizes to IS-2.15 suppressor factor-encoding nucleic acids, and polypeptides or proteins specifically bound by antisera to IS-2.15 suppressor factor, e.g., antisera to the active site or binding domain of IS-2.15 suppressor factor.

The invention are also includes polypeptides that differ from the naturally-occurring suppressor factor by substitution of one amino acid for another of the same class, or that differ by one or more non-conservative amino acid substitutions, deletions, or insertions, located at positions of the amino acid sequence that do not destroy the biological activity of the polypeptide. Amino acids of the same class are ones that share characteristics of hydrophobicity, charge, pKa, or other conformational or chemical properties (e.g., valine for glycine, arginine for lysine, etc.)

As used herein, the term "substantially pure" describes a protein, e.g., an IS-2.15 suppressor factor protein or polypeptide, that has been separated from

components, i.e., the components of a eukaryotic cell. Typically, a protein is substantially pure when at least 60%, more preferably at least 75%, more preferably at least 90%, and most preferably at least 99%, of the total material (by volume, by wet or dry weight, or by mole per cent or mole fraction) in a sample is the compound of interest. Purity can be measured by any appropriate method, e.g., in the case of polypeptides by column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis.

A "purified nucleic acid", as used herein, refers to a nucleic acid sequence or fragment that is not associated with the sequences that flank it in a naturally occurring state, e.g., a DNA fragment that has been removed from the sequences that are adjacent to the fragment, e.g., the sequences adjacent to the fragment in its normal site in the genome. The term also applies to nucleic acids that have been substantially purified from other components that naturally accompany it in the cell, and also applies to cDNA and synthetic nucleic acids.

The properties of the suppressor factor of the invention render it and its biologically active analogs and fragments useful in a number of therapeutic and diagnostic applications. In therapy in particular, the factor, because of its immunosuppressive effects, may be useful in the treatment of autoimmune diseases such as lupus, type 1 diabetes, and rheumatoid arthritis. The factor may also be used to inhibit transplant rejection and graft versus host disease (GVHD) following transplantation. When used for this purpose, the suppressor factor will be administered generally by the same regimens, and in the same dosage range, as current commercially-available immunusuppressive agents, with the provision that, because the suppressor factor of the invention is a protein, it will preferably be

administered intravenously rather than orally. A nucleic acid encoding the suppressor factor of the invention may be used to protect cells against autoreactive T-cells.

In another aspect, the invention features, a method for inhibiting rejection of a transplanted tissue in a mammal, involving (a) introducing into a cell of the tissue DNA encoding the immunosuppressive protein and (b) transplanting the tissue into the mammal so that the immunosuppressive protein is expressed (and preferably secreted) by the cell of the tissue. The immunosuppressive protein is, according to the invention, produced in the localized anatomical region where it is required, i.e., in the vicinity of the transplanted tissue, but is not administered systemically to the animal, so that generalized systemic immunosuppressive effects are not produced.

The method can be used to inhibit rejection of both allografts and xenografts, e.g., transplanted organs such as heart, kidney, liver and lung, and tissues such as bone and skin.

The invention can employ DNA which encodes any immunosuppressive protein. Examples of suitable proteins are interluken-10 (IL-10) transforming growth factor $\beta$ (TGF-$\beta$) and the suppressor factor described above produced by human T-cell clones such as IS-2.15.

Expression of the immunosuppressive protein in the cell of the transplanted tissue can be controlled by regulatory sequences which cause constitutive expression, or alternatively, expression can be controlled by regulatory sequences which are inducible; in one preferred embodiment, the regulatory sequence controlling expression of the immunosuppressive protein is inducible by a compound which stimulates an immune response. Thus, for example, the promoter can be inducible by IL-2 which stimulates proliferation of immune cells which tend to

cause rejection of the transplanted organ; thus, when the immune system is being stimulated to cause a rejection, one of the factors causing that stimulation at the same time, turns on the gene for the immunosuppressive protein, countering rejection. Alternatively, the promoter controlling transcription of the immunosuppressive protein gene is inducible by a foreign antigen of the transplanted tissue itself; in this case, as well, the same component which tends to cause rejection also turns down expression of the protein inhibiting rejection.

Other features and advantages of the invention will be apparent from the following detailed description thereof, and from the claims.

## Detailed Description

The drawings are first described.

### Drawings

Fig. 1. is a Southern blot of products of a reverse transcriptase-polymerase chain reaction (RVT-PCR). RVT-PCR transcripts of IL-2 in islet cell grafts and spleens on day 8 post engraftment. Four mice were treated with DAB486-IL-2 (lanes 1-4) and four with anti-CD3 mAb (lanes 5-8) for 8 days. All harvested tissue was snap frozen in liquid nitrogen and total RNA extracted by the GCN method. Five μg of total RNA was used as starting material for RVT-PCR. Products were size separated on a 1.5% agarose gel to confirm the product size and blotted to a nylon membrane and probed with a cDNA labelled with $^{32}P$. The blots were then scanned using the PHOSPHOR IMAGER system (Molecular Dynamics Inc.) (NK Normal kidney).

Fig. 2 is a Southern blot of PCR products. PVT-PCR transcripts of IFN Gamma in 5 rejecting grafts (lanes 9-13). 4 grafts treated with DAB-IL-2 (lanes 1-4) for 8 days and 4 grafts treated with anti-CD3 mAb (lanes 5-8)

for 8 days. All grafts were harvested on day 8 post transplant, snap frozen in liquid nitrogen and processed for total RNA. Two $\mu g$ of total RNA was used for RVT-PCR. Products were run on a 1.5% agarose gel and blotted to a nylon filter then hybridized with an IFN Gamma cDNA probe labelled with $^{32}P$. The blots were then scanned using the PHOSPOR IMAGER system (Molecular Dynamics Inc.) (NK normal kidney. BL blank.).

Fig. 3 is a Southern blot of PCR products. RVT-PCR products of coamplified GRANZYME B and TCR C$\alpha$ mRNA transcripts from spleens 1, 4, 12, 21 days post transplant, in rejecting model of pancreatic islet cell transplantation, and CON A stimulated spleen cells. One $\mu g$ of total RNA per sample was used as starting material. The products were size separated on a 2.5% agarose gel, blotted to a nylon membrane and cohybridized with $^{32}P$ labelled cDNA probes for GRANZYME B and TCR C$\alpha$. Using the PHOSPHOR IMAGER and IMAGEQUANT software (Molecular Dynamics Inc.) the bands were quantitated and expressed as a ratio of GRANZYME B to TCR C$\alpha$ as well as a percentage relative to the CON A control. As can be seen there was a progressive increase in the transcription level of GRANZYME B during rejection relative to TCR C$\alpha$.

Fig. 4a is an autoradiograph showing the results of CAT assays of extracts prepared from U-937 cells transfected with a 1.2 kb *BamH1-Xho1* IL-6-CAT construct containing wild-type (WT) (lane 3, 6, 9, 12, 15, and 18). mutant M1 (lanes 2, 5, 8, 11, 14, and 17), or mutant M2 (lanes 4, 7, 10, 13, 16 and 19) kB sites as illustrated in Fig. 1. The cells were either uninduced (lanes 2 to 4) or stimulated 33 h after transfection with 10 $\mu g$ of LPS per ml (lanes 5 to 7). 2 $\mu g$ of PHA per ml (lanes 8 to 10). 100 $\mu g$ of double-stranded RNA poly(IC) (Pl-pC) per ml (lanes 14 to 16), or 100 units of IFN-$\gamma$ per ml (lanes 17 to 19) for 19 h.

Fig. 4b is a graph of relative CAT activity after induction by various stimuli in U-937 cells. Acetylated and nonacetylated forms of chloramphenicol from the assay were quantitated by liquid scintillation counting. Values are represented as fold induction of the wild-type (WT) or mutant IL-6 promoter over CAT activity expressed in uninduced cells. Similar results were obtained in three independent experiments. Induction of IL-6-CAT expression in U-937 cells depends on an intact kB site.

Fig.5 is a set of three graphs illustrating the effect of transforming growth factor (TCR) crosslinking on proliferation of the IS-2.15 clone. Each point represents the mean of triplicate determinations ± SEM. As a positive control, the same experimental procedure was undertaken using $10^5$ NOD spleen cells.

Fig.6 is a pair of graphs illustrating that IS-2.15 supernatant specifically inhibits IL-2 dependent proliferation in IL-2 dependent murine T-cell lines. Fig.6A shows the effect of supernatant (50% final dilution) from 3 different clones upon T-cell proliferation. Each point represents mean ± SEM of triplicate determinations. Fig.6B represents the effect of titrating IS-2.15 supernatant versus different concentrations of IL-2.

Fig. 7 is a graph illustrating that the inhibitory activity of IS-2.15 is not reversible. Results represent cpm ± 1 SD of triplicates.

Fig. 8 is a graph illustrating that IS-2.15 supernatant does not modify IL-2Rα expression on HT-2 cells.

Fig. 9 is a graph illustrating that the molecular weight of IS-2.15 supernatant suppressor activity is between 10 and 30 KD.

Fig. 10 is a diagram of plasmid, IL-10/INSULIN-pSf3. It is 6.05 kb in size. The IL-10 gene was cloned

into the plasmid, pSf3, and it is under the transcriptional control of the insulin promoter.

Fig. 11 is a diagram of plasmid, IL-10/IgH-pTZ19R. It is 5.82 kb in size. The IL-10 gene was cloned into the plasmid, pTZ19R, and it is transcribed under the control of the IgH enhancer/fos promoter.

Fig. 12 is a diagram of plasmid, IL-10/IL-6P-pTZ19R. It is 6.27 kb in size. The IL-10 gene was cloned into the pTZ19R plasmid and it is transcribed under the control of the IL-6 promoter.

Fig. 13 is a diagram of plasmid, IL-10/HTLVI-pcD-SRα. It is 5.10 kb in size. The IL-10 gene was cloned into the pcD-SRα plasmid and it is transcribed under the control of the HTLV1 LTR.

Fig. 14 is a diagram of plasmid, IL-10/IL-2-pTZ19R. It is 5.50 kb in size. The IL-10 gene was cloned into the pTZ19R plasmid and it is transcribed under the control of the IL-2 promoter.

Novel Suppressor Factor and Novel Method of Inhibiting Rejection of a Transplanted Tissue.

The following will now be described in detail: I. Isolation and characterization of the novel suppressor factor, and II. Inhibition of rejection of transplanted tissue.

I. Novel Suppressor Factor. There follows a detailed description of the transplant model in which the immunosuppressive effects of the suppressor factor of the invention were observed, followed by a description of the cells from which the factor was isolated, the procedure used for isolation, and the experiments in which immunosuppression was observed.

Allogeneic transplant model. We have investigated murine islet cell transplants as a model to study allograft rejection (Pankewycz, et al., 1989 Transplantation 47:318; Mackie, et al., 1990

Transplantation 49:1150) and performed allogeneic pancreatic islet cell transplants according to described techniques herein incorporated by reference (Gotoh, et al., 1985 Transplantation 40:437). Pancreatic islets harvested from DBA/2 ($H-2^d$)mice are transplanted under the renal capsule of 8-10 wk old $B6AF_1$ ($H-2^{b/k, d}$) mice previously rendered diabetic by the beta cell toxin, streptozotocin (250mg/kg ip). Following successful transplantation, blood glucose returns to normal (<200mg/dl) within 7 days. Blood glucose levels are assessed every other day throughout the first 50 days post-transplantation, then twice weekly through the next 50 days. Rejection is defined as hyperglycemia in excess of 300 mg/dl or 3 consecutive days of blood glucose >250mg/dl.

Unmodified graft rejection. Animals are sacrificed at days 4, 8, or 12. Histological examination of day 4 pancreatic islet allografts showed early leukocyte infiltration but well preserved islet morphology. Day 8 allografts showed a massive leukocyte infiltrate with severe islet injury; however, the islets were still discernable. By day 12 the islets were completely destroyed. There was early organization of the cellular exudate, and the leukocyte infiltrate was less intense than observed on day 8. Immunoperoxidase staining of day 12 allograft specimens showed that most infiltrating leukocytes were macrophages, and expressed endogenous peroxidase.

Tolerance induction. Bluestone and his colleagues first reported that high doses of hamster and anti-mouse CD3 mAb caused profound immunosuppression (Hirsch, et al, 1988 J. Immunol. 140:3766). It was noted that lower doses, closer to clinically applied doses, produced tolerance in many, but not all, islet allograft recipients (Mackie, et al., 1990 Transplantation

49:1150). In a new study using the same high total dose employed by Bluestone but administered over a 3d period, permanent engraftment of 90% of DBA/2 into $B6AF_1$ islet graft recipients was obtained. Daily treatment with a recombinant IL-2 diphtheria toxin related fusion protein ($DAB_{486}$-IL-2) also causes a 50% incidence of graft tolerance (Pankewycz, et al., 1989 <u>Transplantation</u> 47:318). Twice daily therapy results in 90% incidence of tolerance.

<u>PCR based identification of activation associated transcripts</u>. PCR can be used to identify activation associated transcripts. The PCR technique was adapted for mRNA phenotyping from tissues that were snap frozen in liquid nitrogen in the following fashion: (a) total cellular RNA was isolated using the cesium chloride modification of the guanidine isothiocyanate method, (b) first-strand cDNA was synthesized using oligo (dT) primer and M-MLV reverse transcriptase, and (c) the cDNA was amplified (25 cycles) with sequence specific oligonucleotide primer-pairs and thermostable DNA polymerase (Taq DNA polymerase), using a DNA thermal cycler. Each sample as co-amplified with a $\beta$-actin oligonucleotide primer as an internal control. The PCR products were analyzed by agarose gel electrophoresis for the predicted fragment size and for specificity by Southern hybridization using $^{32}P$ randomly primed partial length DNA probes. In each case, the radiolabelled cDNA probes identified a single characteristic band when applied to RNA-revived from ConA + RNA activated splenic sutures. We have determined the number of cycles required for attainment of the linear portion of the radioactivity curve following hybridization with radiolabeled probes. For detection of each of the mRNAs, 25 cycles f PCR amplification was adequate for this purpose. The antisense and sense primers hybridize to

sequences encoded by different exons (Table 1). This precaution reduces the possibility that DNA, rather than RNA, sequences are unwittingly selected for amplification and confused with RNA based amplification. In each experiment, one control sample lacks reverse transcriptase in order to identify preparations with contaminant DNA. The relative abundance of each amplified product was semiquantitatively evaluated using the PHOSPHOR IMAGER system (Molecular Dynamics, Inc.).

Correlation of allograft transcriptional activity with histology. RNA was isolated from allografts, lymphoid tissue and non-lymphoid tissue at days 1, 4 (the time of maximal cellular infiltration), 8, 12 (the time of complete graft destruction) and day 19. Peak IL-2 and IFNγ gene transcription in the allograft closely correlated in time with the peak of leukocytic infiltration (Figs. 1, 2 and Table 2). IL-2 and IFNγ transcripts were expressed on day 8, but not at day 1, and only sporadically at days 4 and 12. Whereas IL-2 transcripts were detected in all rejecting allografts at day 8 post-transplant, IL-4 transcripts, although present in some grafts, were barely detectable at any time point. Granzyme B transcripts were detected in most allografts even as early as day 1 in some grafts. Nonetheless, co-amplification of granzyme B and TCR Cα sequences revealed that the ratio of granzyme B to TCR Cα rose progressively through days 1 to 12 (Fig. 3). The ratios of granzymes B to TCR Cα transcripts were compared to those obtained from a 72h Con A activated B6AF$_1$ spleen cell culture. β-actin transcripts were detected in all specimens. Table 2 shows the pattern of IL-2, IFNγ, IL-4 and grandzyme B transcription at Day 8 in acutely rejecting mice. It is noteworthy that detection of IL-2 and granzyme B transcripts in the allograft foreshadowed their appearance in the lymph nodes and spleen. However, the

data also indicates that the pattern of transcriptional activity in the spleen at day 8 closely reflects the pattern of transcriptions in the allograft.

Molecular characterization of allograft tolerance. Animals are treated with immunosuppressive regimes (e.g., anti CD3, DAB $_{486}$-IL-2, or IL-2 Fc) to induce permanent engraftment ["tolerance"] and the transcriptional activity is compared with activity in unmodified graft rejection and syngeneic grafts. Animals are sacrificed at sequential time points during the induction and maintenance phase of graft tolerance and mRNA isolated from the graft, spleen and lymph node.

Transcriptional activity in unmodified, anti-CD3 or DAB$_{486}$-IL-2 treated hosts. A reduction in proportion of grafts/spleen bearing detectable IL-2 and IFNγ mRNA was evident in hosts receiving tolerogenic therapies (Table 2). These data suggest that TH1 subset activation is interrupted by tolerogenic therapies. The proportion of tissues bearing detectable granzyme B and IL-4 transcripts was not altered by treatment. Our preliminary results indicate that IL-10 transcription is evident in all grafts from unmodified and treated hosts. This data indicates that inactivation/destruction of TH1 cells may be closely linked to tolerance induction, and the balance between expression of pro-inflammatory (IL-2, IFNγ) and anti-inflammatory transcripts (IL-10) is markedly altered in hosts receiving tolerogenic therapy.

Pathology and transcriptional events in pancreatic islet cell allografts during rejection and tolerance induction. Using a model of mouse pancreatic islet cell transplantation and the RVT-PCR technique, intragraft and splenic expression of IL-2, IFNγ, IL-2R α, IL-4, and granzyme B mRNA was studied. An analysis of IL-10 mRNA was initiated. In a totally MHC mismatched transplant (DBA2/J-B6AF1/J), the graft is completely rejected by day

19. Mice were transplanted and sacrificed at 1, 4, 8, 12 and 19 days post transplant. Maximal mononuclear cell infiltration of the allografts occurred at day 8. The peak expression of IL-2 and IFNγ mRNA also occurred at day 8 while meager IL-4 transcripts were detected in some grafts at this point. Peak expression of granzyme B occurred at days 8-12. In a second set of experiments, transplanted mice were treated either with anti-CD3 mAb or an IL-2 toxin fusion protein (DAB/486-IL-2) with doses that we have shown to significantly delay rejection and to induce tolerance in at least 90% of mice. Treated mice were sacrificed 8 days post transplant for RNA extraction. IL-2 and IFNγ transcription was markedly reduced. Preliminary data indicate that tolerogenic therapies do not block IL-10 expression.

Islet cell graft infiltrating T-cell lines and T-cell clones. Using the methods described by Fitch and Gajewski (Fitch, 1991 Current Protocols in Immunology. 3.13.1-3.13.11), both rIL-2 (80 U/ml) and rIFNγ (4000 U/ml) or a mixture of rIL-2 and Con A supernatants to derive outgrowths of islet graft infiltrating T cells were used. The IL-2 and IFNγ mixture favors TH1 cell growth while the latter mixture favors TH2 cell growth (Fitch, 1991 Current Protocols in Immunology. 3.13.1-3.13.11). The outgrowths are then cultivated in media that favors the maintenance of TH1 or TH2 cells. Other clones are derived using only rIL-2 and donor strain islets. To date we have derived at least two T-cell lines cultivated under TH1- and one cultivated under TH2- promoting growth media from day 2, 8, 12, 19 unmodified and DAB\486-IL-2 treated hosts. Aliquots of these lines have been frozen and stored. A limiting dilution method (MacDonald, t al., 1990 Immunol. Rev. 51:93) to derive T-cell graft infiltrating clones from day 8 unmodified and DAB\486-IL-2 treated hosts was used.

The media used to selectively propagate TH1 and TH2 clones does yield a high proportion of CD4+ clones while use of a protocol utilizing IL-2 and islet cell cultures primarily yields CD8+ T-cells.

Regulatory elements of the IL-6 promoter.

Interleukin-6 (IL-6) is a cytokine released by a variety of cells including macrophages, endothelial cells, fibroblasts, T cells, glial cells and B cells in response to a variety of stimuli (Kishimoto, 1989 Blood 74: 1-10). In particular IL-6 gene expression is stimulated by all substances that trigger an immune response and inflammation, the same agents released during acute rejection (Kishimoto, 1989 Blood 74: 1-10). The IL-6 promoter is composed of a variety of overlapping regulatory elements (Kishimoto, 1989 Blood 74: 1-10). The IL-6 promoter has been cloned and inserted upstream of the chloramphenicol acetyltransferase gene. A 1.2 kb fragment of the 5′ flanking region of the IL-6 gene (Ray, et al., 1988 Proc. Natl. Acad. Sci USA 85:6701-6705; Yasukawa, et al., 1987 Embo J. 6:2939-2945) contains all the necessary elements for induction of the IL-6 gene. Analysis of the promoter region of the IL-6 gene revealed the presence of a putative binding site for the transcription factor NF-kB (Libermann, et al., 1990 Mol. Cell. Biol. 10:2327-2334).

Putative regulatory elements of the IL-6 promoter. To characterize the functional role of the putative NF-kB binding site, mutations were introduced into the IL-6kB site using synthetic oligonucleotides and the gapped/heteroduplex method (Stewart, et al., 1988 Biotechniques 6:511-518) and examined for their effects on inducibility of the IL-6 gene. Enhancer activity was measured as the ability of the different enhancer constructs to induce transcription of the chloramphenicol acetyltransferase (CAT) gene after transient expression

in a variety of cell types (Libermann, et al., 1990 Mol. Cell. biol. 10:3155-3162; Gorman, et al., 1982 Mol. Cell. Biol. 2:1044-1051; Gilman, et al., 1986 Mol. Cell. Biol. 6:4305-4316). Transfections of U-937 monocytic cells, Jurkat T cells and HeLa cells were carried out using the DEAE-dextran method (Pierce, et al., 1988 Proc. Natl. Acad. Sci USA 85:1482-1486). The apparent NF-kB binding site is an indispensable component of the IL-6 control region. The IL-6 promoter kB site binds NF-kB and its alteration abolishes inducibility of chimeric genes driven by the IL-6 promoter (Fig. 4) (Libermann, et al., 1990 Mol. Cell. Biol. 10:2327-2334). It appears that the ability of various agents including LPS, PMA, TNF-α and dsRNA to induce IL-6 expression is a consequence of their ability to activate NF-kB. A second enhancer element, NF-IL-6, was also shown to respond to IL-1, IL-6, TNFα, and LPS (Isshiki, et al., 1990 Mol. Cell. Biol. 10:2757-2764) and recent evidence suggests that NF-kB interacts cooperatively with the NF-IL-6 factor (Mukaida, et al., 1990 J. Biol. Chem. 265:21128-21133). Mutations in either elements will abolish inducibility. Ray et al. identified an additional element, MRE, that overlaps with a potential cAMP responsive element (CRE) (Ray et al., 1989 Mol. Cell. Biol. 9:5537-5547). The MRE is apparently induced by phorbol ester, IL-1, TNF-α and forskolin (Ray et al., 1989 Mol. Cell. Biol. 9:5537-5547). In addition, two putative glucocorticoid response elements and an AP-1 binding site have been identified in the IL-6 promoter (Kishimoto, 1989 Blood 74:1-10). Potential binding sites for HLH and ets related transcription factors are also present, as well as a putative binding site for factors of the GATA gene family (Martin, et al., 1990 Nature 344:444-447). The IL-6 promoter can be used as an inducible promoter for IL-10 and TGF-β expression.

Cells. The anergic T-cell clone IS-2.15 is a recombinant human IL-2 (rIL-2) dependent, non-cytolytic, CD8+, Vβ11+ clone propagated from the islets of 2 month old euglycemic male NOD mice.

The T-cell clone, IS-2.15, was deposited with the American Type Culture Collection on February 26, 1993, and bears the accession number ATCC no.__________________. Applicants acknowledge their responsibility to replace the plasmid loose viability before the end of the term of a patent issued hereon, and their responsibility to notify the American Type Culture Collection of the issuance of such a patent, at which time the deposit will be made available to the public. Prior to that time the deposit will be made available to the Commissioner of Patents under the terms of CFR §1.14 and 35 USC §112.

Transfer of IS-2.15 T-cells into prediabetic NODs prevents rapid induction of diabetes of accelerated diabetogenic autoimmunity (Pankewycz, O., et al., 1991, *Eur. J. Immunol.* 21:873). The suppressive effects of IS-2.15 are characterized in the following detailed description.

H-4, G-3, #3 and #5 cells are CD3+, CD8+ islet-infiltrating T-cell clones isolated from the islets of 2 month old euglycemic NOD mice as previously described (Pankewycz, O., et al., 1991, *Eur. J. Immunol.* 21:873). HT-2 (Watson, J., 1979, *J. Exp. Med.* 150:151) and CTLL-2 (Gillis, S., et al., 1977, *Nature* 268:154.) murine IL-2/IL-4 dependent T-cells were obtained from Dr. D. Perkins (Brigham and Women's Hospital, Boston, MA) and the American Type Culture Collection (Rockville, MD), respectively.

T-cells were cultured in RPMI-1640 media (Gibco, Grand Island, NY) supplemented with 10% (vol/vol) fetal bovine serum, 100μg/ml streptomycin, 100 u/ml penicillin,

10 μM HEPES, 1 μm sodium pyruvate, $10^{-5}$ M 2-mercaptoethanol and 1% minimal essential amino acids (complete RPMI medium). In some cultures irradiated (3,000 rads) syngeneic splenic cells were plated with IS-2.15, G-3 and H-4 cloned T-cells. T-cell growth factor (TCGF) was added to some HT-2 and CTLL-2 cell cultures.

Supernatant from NOD T-cell clones

T-cell clones were isolated from feeder cells by Lympholyte M (Cedarlane Lab. Hornby, Ontario, Ca.) gradient separation and placed in culture at a concentration of $10^6$ cells/ml in complete RPMI medium for 24 hours. After this period, T-cells were pelleted by centrifugation, and the supernatant was aliquoted in siliconized tubes and stored at -70°C.

Proliferative responses against immobilized mitogenic mAbs Fifty x $10^3$ cloned T-cells were cultured in round bottom 96-well plates in 0.2 ml of complete RPMI medium alone or with 5 x $10^5$ irradiated (3,000 rads) syngeneic spleen cells and different concentrations of immobilized anti-CD3 or anti-Vβ11 mAbs. Wells were coated with mAbs for 2 hours at 37°C. Cells were cultured in the mAb coated plates for 48 hours, pulsed with 1 μCi [$^3$H]thymidine during the last 6 hours of culture, harvested with a semiautomated cell harvester (PHD, Cambridge, MA), and cellular [$^3$H]thymidine incorporation was measured by liquid scintillation counting.

Proliferative responses against syngeneic pancreatic islets Islets of Langherhans from NOD mice were obtained as previously described (Pankewycz, O., et al., 1991, *Eur. J. Immunol.* 21:873), irradiated (3,000 rads) and placed in flat-bottom 96-well plates with $10^5$ T-cells in complete RPMI. Cells were cultured for 6 days, pulsed with 1 μCi [$^3$H]thymidine during the last 6 hours of culture, harvested with a semiautomated cell harvester (PHD, Cambridge, MA), and cellular incorporation of

[$^3$H]thymidine was measured by liquid scintillation counting.

Detection of suppressor activity

HT-2 or CTLL-2 cells were cultured at a concentration of $10^5$ cells/well in a final volume of 0.2 ml complete RPMI media ± rIL-2 ± supernatants of NOD T-cell clones and ± anti-TGF-$\beta$1 or anti-TGF-$\beta$2 mAbs. After 20 hours of culture, each well was pulsed with 1 $\mu$Ci [$^3$H]thymidine, incubated for an additional 4 hr, and harvested with a semiautomated cell harvester (PHD, Cambridge, MA). Tritiated thymidine incorporation was measured by liquid scintillation counting. Suppressor activity was calculated according to the following formula: % suppressor activity: [experimental samples (supernatant + IL-2) - negative control (no IL-2)/[positive control (IL-2 alone) - negative control]. Results were given as the mean of triplicate cultures. SEM did not exceed 12.5% of means. Similar results were found for HT-2 or CTLL-2 cells.

Bioassay for Transforming Growth Factor $\beta$ (TGF-$\beta$)

Mink lung epithelial (CCL-64) TGF-$\beta$ indicator cells were used as previously described (Danielpour, D., et al., 1989, *J. Cell. Physiol.* 138:79). Briefly, $10^5$ cells/ml were placed in 24 well plates with culture media containing T-cell supernatants and cultured for 22 hours. The cells were then pulsed with 0.5 $\mu$Ci/well of [$^3$H]thymidine for 2 hours, and [$^3$H]thymidine incorporation was measured by liquid scintillation counting. A TGF-$\beta$ standard curve was constructed using TGF-$\beta$ indicator cells cultured with serial dilutions of a known amount of TGF-$\beta$.

Flow cytometric analysis

HT-2 cells (2.5 x $10^5$) were suspended in 50 $\mu$l Hank's balanced salt solution containing 0.1% sodium azide and 20% (vol/vol) horse serum. The cells were

incubated for 30 minutes at 4°C with 0.2 μg of anti-IL-2Rα mAb, washed and incubated with anti-rat IgG-FITC conjugated 30 minutes at 4°C. Subsequently, the cells were washed, resuspended in 2% paraformaldehyde and analyzed using a Becton and Dickinson FACS analyzer.

Partial size purification

Supernatants harvested from 24 hour cultures of IS-2.15 T-cells grown in complete RPMI without fetal bovine serum were loaded into 10, 30 and 100 KD membrane Centricon microconcentrator tubes (Amicon, Danvers, MA) with different membrane size cut-offs (10, 30 and 100 KD) and centrifuged at 6,000 RPM for 60 minutes at 25 °C. The concentrates and filtrates were tested for biological activity as described previously.

RNA extraction and Polymerase-Chain reaction (PCR) procedure Cytoplasmic RNA was extracted from T-cell clones by the NP-40 lysis method. Two micrograms of RNA were reverse transcribed into cDNA using random hexamer oligo$(dN)_6$ as primer (BRL) and AMV reverse transcriptase (Promega, Madison, WI) in a 50 μl reaction. Ten microliters of the cDNA was amplified by PCR. PCR conditions implemented in a 50 μl reaction were as follows: 75-750 pmol of each primer (see below), 200 μM each dGTP, dATP, dCTP and dTTP (Perkin-Elmer/Cetus, Emeryville, CA), 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, and 2 units Taq DNA polymerase ("AmpliTaq", Perkin-Elmer/Cetus, Emeryville, CA). The following primers were used: IL-2 sense primer 5'-TGATGGACCTACAGGAGCTCCTGAG-3' (nucleotides 203'-227'); IL-2 antisense primer 5'-GAGTCAAATCCAGAAACATGCCGCAG-3' (nucleotides 370'-346'); IL-4 sense primer 5'-CGAAGAACACCACAGAGAGTGAGCT-3' (nucleotides 231-255); IL-4 antisense primer 5'- GACTCATTCATGGTGCAGCTTATCG-3' (nucleotides 411'-387'); IL-6 sense primer 5'-TGGAGTCACAGAAGGAGTGGCTAAG-3' (nucleotides 581'-605');

IL-6 antisense primer 5′-TCTGACCACAGTGAGGAATGTCCAC-3′ (nucleotides 735′-711′); IFN-γ sense primer 5′-AGCGGCTGACTGAACTGAACTCAGATTGTAG-3′ (nucleotides 841′-865′); IFN-γ antisense primer 5′-GTCACAGTTTTCAGCTGTATAGGG-3′ (nucleotides 1084′-1061′); TNF-α sense primer 5′-GGCAGGTCTACTTTGGAGTCATTG-3′ (nucleotides 820′-843′); TNF-α antisense primer 5′-ACATTCGAGGCTCCAGTGAATTCCAG-3′ (nucleotides 1127′-1102′); TGF-β sense primer 5′-AAGTGGATCCACGAGCCCAA-3′ (nucleotides 1277′-1298′); TGF-β antisense primer 5′-CTGCACTTGCAGGAGCGCAC-3′ (nucleotides 1521′-1502′), as previously described (Murray, L.J., et al., 1990, *Eur. J. Immunol.* 20:163). Reactions were incubated in a Perkin-Elmer/Cetus DNA thermal cycler for 45 cycles (denaturation 30 seconds, 95°C; annealing 30 seconds, 56°C; extension 60 seconds, 72°C). Fifteen microliters of the reaction product was electrophoresed through a 1.2% agarose gel and transferred onto a nylon membrane by capillary action (Zeta-probe, Bio-Rad, Richmond, CA). Blots were hybridized at 65°C for 18 hours with 1 mM EDTA, 0.5 M $NaH_2PO_4$ (pH 7.2) and 7% sodium dodecyl sulfate (SDS), and radiolabeled with probes for murine rIL-2 (90 bp probe inserted in pBS) (Kasima, N., et al., 1985, *Nature* 313:401), IL-4 (1 kb probe inserted in pXM) (Lee, F., et al., 1986, *PNAS* 83:2063), IL-6 (1 kb probe inserted in pXM) (Chiu, C.P., et al., 1988, *PNAS* 85:7099), IFN-γ (643 bp probe inserted in pms10) (Gray, P.W., et al., 1983, *PNAS* 80:5842), TNF-α (480 bp probe inserted in p-mTNF-1) (Fransen, L. et al., 1985, *Nucl. Ac. Res.* 13:4417) and human TGF-β (2.14 kb probe inserted in pBR327) (Derynck, R., et al., 1986, *J. Biol. Chem.* 261:4377). After hybridization the blots were washed twice at 65°C with 1 mM EDTA, 40 mM $NaH_2PO_4$ (pH 7.2) and 5% SDS for 30 minutes, and twice with 1 mM EDTA, 40mM $NaH_2PO_4$ (pH 7.2) and 1% SDS for 30 minutes at 65°C. Blots

were then exposed to Kodak X-AR film at -70°C for 6-24 hours. Results of the analysis of cytokine gene expression are described below.

T-cell receptor (TCR) crosslinking did not induce proliferation in an IS-2.15 clone.

To determine whether IS-2.15 is an anergic or normally responsive T-cell clone, culture plates were coated with mitogenic anti-TCR/CD3 complex and cellular proliferation was measured. Unlike NOD spleen cells, IS-2.15 T-cells did not proliferate in response to anti-CD3 or anti-Vβ11 mAbs (Fig.5). Increasing the concentration of either the mAbs (Fig.5A and Fig.5B) or the concentration of responder cells (Fig.5C) failed to produce mitogenesis. These findings have been corroborated in each of three subsequent experiments. To determine whether this unresponsiveness to anti-TCR/CD3 mAbs was merely caused by a delay in proliferation the culture period was prolonged to 5 days. A failure to proliferate in response to anti-CD3 or anti-Vβ11 mAbs was also noted at 5 days.

IS-2.15 clone proliferates, albeit slowly, to syngeneic spleen cells or pancreatic islets.

To assess whether IS-2.15 T-cells are autoreactive $5 \times 10^5$ IS-2.15 T-cells/ml were co-cultured with 50-70 irradiated syngeneic NOD islets/ml or $10^6$ spleen cells/ml in a final volume of 200 μl. Although IS-2.15 T-cells responded with a far lower proliferative rate than many other organ-specific islet infiltrating T-cell clones (Pankewycz, O., et al., 1991, *Eur. J. Immunol.* 21:873), IS-2.15 T-cells do proliferate, albeit weakly, in response to islets. Table 3 shows a representative, i.e. one of three, experiment.

IS-2.15 supernatant specifically inhibits rIL-2 dependent Proliferation in rIL-2 dependent murine T-cell lines.

To investigate the possibility that IS-2.15 T-cells produce an immunosuppressive factor, the ability of IS-2.15 to block IL-2 stimulated proliferation of murine T-cells was tested. Two different murine IL-2 dependent T-cell lines, CTLL-2 and HT-2, were used as defined model systems for this analysis. When either of these T-cell lines are pre-incubated with IS-2.15 supernatant, their proliferative response to rIL-2 was abrogated, indicating that a suppressor or anti-proliferative substance is present in this supernatant (Fig.6A). To examine whether this inhibitory substance was produced by other islet infiltrating NOD T-cell clones, supernatants from two additional islet-infiltrating clones were analyzed. Neither clone produced a substance that inhibited the proliferative response of CTLL-2 or HT-2 cells to rIL-2. Supernatants from cloned G-3 cells did not modify rIL-2 stimulated proliferation, whereas supernatants from cloned H-4 cells actually amplified rIL-2 dependent T-cell proliferation. Titration of IS-2.15 suppressor activity showed that supernatant dilutions of 1/16 were suppressive (Fig.6B).

Although IL-2 indicator cells (CTLL-2 AND HT-2) cultured with IS-2.15 supernatant and rIL-2 do not proliferate, HT-2 cells remain viable after interaction with the supernatant as determined by dye trypan blue exclusion. Viability of cells cultured for 24 hours with rIL-2 was 99% with or without addition of IS-2.15 supernatant.

<u>IS-2.15 supernatant inhibitory activity is not due to TGF-β</u>

Since TGF-β is the only cytokine known to directly block the response of T-cells to IL-2, the capacity of neutralizing anti-TGF-β1 and anti-TGF-β2 antibodies to restore proliferation of HT-2 cells cultivated with rIL-2 and IS-2.15 T-cell supernatants was tested. Anti-TGF-β1

and anti-TGF-$\beta$2 antibodies, used at neutralizing concentrations as described elsewhere (Danielpour, D., et al., 1989, *J. Cell. Phisiol.* 138:79), failed to restore rIL-2 dependent proliferation of HT-2 cells. Moreover, TGF-$\beta$ bioactivity was not detected in IS-2.15 T-cell supernatants using TGF-$\beta$ dependent epithelial lines as indicator cells. It is doubtful that the active moiety is TGF-$\beta$.

Inhibition of rIL-2 stimulated proliferation of HT-2 cells is not readily reversible and is not due to downregulation of the IL-2 receptor (IL-2R$\alpha$).

Saturating concentrations of rIL-2 do not restore IL-2 dependent proliferation of HT-2 cells cultured with IS-2.15 supernatant (Fig.5A). The mechanism of this inhibitory activity was investigated. HT-2 cells were cultured with rIL-2 (50 u/ml) ± IS-2.15 supernatant (10%, vol/vol) for 24 hours. HT-2 cells were washed twice and recultured for an additional 24 hours with different concentrations of rIL-2. HT-2 cells that were not pretreated with IS-2.15 supernatant responded to rIL-2. In contrast, HT-2 cells pre-cultured for 24 hours with IS-2.15 supernatant and washed did not proliferate in response to rIL-2. Since cell viability was not compromised by IS-2.15 supernatant (99% in the two groups), a cytotoxic effect is not responsible for the anti-proliferative activity (Fig. 7).

In theory, downregulation of IL-2R$\alpha$ expression could account for these effects. Therefore, IL-2R$\alpha$ expression was examined by flow cytometry after culturing HT-2 cells with rIL-2 (50 u/ml)± IS-2.15 supernatant (10%, vol/vol). IS-2.15 supernatant did not inhibit IL-2R$\alpha$ expression in HT-2 cells (Fig. 8).

IS-2.15 supernatant inhibitory factor is a heat-sensitive protein with a molecular weight between 10 and 30 KD.

The inhibitory activity of IS-2.15 upon IL-2 dependent proliferation was completely destroyed by pre-heating IS-2.15 supernatant for 15 minutes at 65°C. Size selective ultracentrifugation procedures indicate that the molecular weight of this substance is between 10 and 30 KD (Fig. 9).

Analysis of cytokine gene expression by PCR.

Messenger RNA was extracted from each of the 3 islet-infiltrating NOD T-cell clones (IS-2.15,H-4,G-3) and amplified via PCR, as described above. TGF-$\beta$ encoding mRNA was detected only in the IS-2.15 clone. This clone also expresses TNF-$\alpha$ mRNA. None of the clones expressed IL-2 encoding mRNA; IL-4 encoding mRNA was detected only in the H-4 islet-infiltrating T-cell clone. This finding correlated with the ability of the H-4 clone supernatant to increase proliferation of the IL-4 and IL-2 sensitive HT-2 line to rIL-2 (Fig.6A). In contrast, the G-3 clone did not express IL-2 or IL-4 encoding mRNA. All 3 clones expressed IL-6 and IFN-$\gamma$ mRNA.

Purification of the IL-2.15 suppressor factor protein.

The IS-2.15 suppressor factor protein or its component polypeptide(s) can be purified using conventional methods of protein purification known to one schooled in the art, e.g., methods including but not limited to precipitation, chromatography, immunoadsorption, or affinity techniques. The polypeptide can be purified from starting material using the IS-2.15 supernatant, an IS-2.15 suppressor factor cDNA or genomic DNA, as described below, or using a recombinant form of either of these DNAs genetically engineered into an overproducing cell line.

Isolation of a human IL-2.15 suppressor factor cDNA and genomic DNA.

A cDNA encoding the IL-2.15 suppressor factor can be isolated from a human expression library by screening

with antibodies to the suppressor factor. Antibodies for screening can be raised in an animal, for example a rabbit. Possible immunogens include but are not limited to 1) a purified fragment of the suppressor factor protein obtained by conventional methods of protein separation as described above, or 2) a partially purified sample of IL-2.15 suppressor factor. The protein sample can be partially purified by fractionating an IL-2.15 supernatant by size, as described above. Antibodies are labeled with a suitable label and are then used as probes to screen an expression library. The cDNA library can be generated by isolating poly($A^+$) mRNA from the IL-2.15 cell line, converting the RNA to double stranded cDNA (Aruffo, A., et al., 1987, *PNAS* <u>84</u>:8573-7) and ligating the cDNA into a λ expression vector, for example λgt11 (Clontech), or into the expression vector pCDNA-1 (Invitrogen, San Diego). Positive clones can be confirmed by testing for the ability to block IL-2 stimulated T-cell proliferation.

The human suppressor factor gene can be cloned by analogous methods, for example by using a human cDNA library. The IL-2.15 cDNA sequences identified above can be used as oligonucleotide probes to obtain the analogous human suppressor factor cDNA or genomic DNA by methods known to those skilled in the art.

## II. Inhibition of Rejection of Transplanted Tissue

There follows a detailed description of the methods by which rejection of organ and tissues can be inhibited according to methods of the invention.

<u>Use</u>. Uses of the suppressor factor protein of the invention are based on its unique properties, in particular, its ability to block IL-2 stimulated T-cell proliferation without being cytotoxic or inhibiting the expression of IL-2 or IL-2 receptor. Uses fall into two main categories: diagnosis and therapy.

Diagnosis. Human patients suffering from autoimmune diseases will be expected to exhibit abnormally low levels of the suppressor factor of the invention in the blood and in the endocrine portion of the pancreas. Assaying blood, other biological fluid samples, or pancreatic tissue samples for the factor can thus provide a means of monitoring therapy and assessing the immune status of patients with autoimmune diseases, e.g., IDDM. Such assays can be carried out by conventional immunoassay methods employing antibodies to the suppressor factor, made by conventional techniques in which a rabbit is immunized with the factor and the resulting antibody harvested and labeled, e.g., with a radioisotope or a fluorescent tag.

Therapy. For use in therapy, the suppressor factor protein of the invention is first purified to homogeneity using conventional techniques. It is then mixed with a pharmaceutical carrier and administered intravenously or by some other standard method, or used to perfuse a graft to be transplanted. The factor can be used to treat autoimmune diseases such as systemic lupus erythematosus (SLE), type 1 diabetes, and rheumatoid arthritis, as well as other disease states involving the immune system such as graft versus host disease. Dosage will be in the range of dosages used for currently available immunosuppressive agents.

A central feature of the invention this aspect of the invention is the design of therapies which specifically suppress the local immune response at the site of potential rejection of the tissue or organ. This strategy, which targets immunosuppression to the specific anatomical region where it is needed, represents an improvement over existing clinical therapies, all of which involve general suppression of the immune response.

The immunosuppressive proteins encoded by the DNA transfecting cells of the transplanted tissue can exert its immunosuppressive effect in any of several ways, for example: (1) the protein itself can exert a direct immunosuppressive effect; (2) the protein can be an enzyme which acts on a substrate to produce a protein or non-protein immunosuppressive compound; or (3) the protein can be an enzyme which is capable of activating a prodrug which, when activated, acts as an immunosuppressive.

Prior to transplantation of the tissue/organ into the mammal, the recombinant gene is introduced into the cells of the tissue/organ. This can be accomplished by *in vitro* transfection of isolated cells of the tissue/organ, or by transfection of the tissue/organ itself. Introduction of the DNA can also be accomplished by transfection of the tissue/organ *in vivo*, for example, by introducing suitably prepared DNA directly into the tissue/organ of the mammal following transplantation. Another method for the introduction of DNA into an organ involves the generation of transgenic animals in which the appropriate genes are expressed in the organ to be transplanted.

Following transplantation, the proteins are expressed and act to inhibit rejection of the transplanted tissue/organ.

<u>Construction of Recombinant Genes</u>

Recombinant proteins capable of mediating inhibition of rejection of a transplanted tissue/organ by modulating the immune response at the site of rejection in a mammal include, but are not limited to, IL-10, TGF-$\beta$ and other IL-2 suppressor factors such as that produced by the T cell clone, IS-2.15. The genes that encode IL-10 and TGF-$\beta$ have been cloned into plasmid vectors as described below. The expression of these genes can be

driven by any one of a number of promoters which are selected because they either control expression of the proteins in an inducible manner, or cause the protein to be expressed constitutively in the cell into which the plasmid is transfected. The recombinant technology required to clone and express such genes is standard in the art and is described in detail, for example, in Sambrook et al. (Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Press, Cold Spring Harbor, NY, 1989).

Promoters. Examples of promoters that confer inducible expression on genes include, but are not limited to, the following.

Publically available promoters that are inducible by factors involved in the immune response: NFκB and NF-IL-6, both of which are induced by IL-1; interferon response element, which is induced by γ-interferon; IL-6 receptor, which is induced by IL-6; CRE, which is induced by prostaglandins.

Promoters that are inducible by exogenous factors: glucocorticoid response element, which is induced by glucocorticoids; retinoic acid response element, which is induced by retinoic acid; NF-κ and NF-IL-6, which are induced by inflammatory agents; Verapamil response element and OS-1 response element, which are induced by verapamil and OS-1; glucose response element, which is induced by glucose; Mouse Mammary Tumor Virus LTR, which is induced by dexamethazone.

Examples of promoters that confer constitutive expression include, but are not limited to, the following:

The human T cell leukemia virus type 1 long terminal repeat; the Rous sarcoma virus long terminal repeat; the Moloney murine leukemia virus long terminal repeat; the simian virus 40 early enhancer/promoter; the

cytomegalovirus immediate early promoter; the β-actin promoter; the islet cell insulin promoter; the B cell immunoglobulin enhancer/promoter; the T cell receptor enhancer promoter that is T cell specific, such as the HTLV-1 promoter, or an endothelial cell specific promoter.

Plasmids or other vectors encoding most of these promoters are commercially available. Where this is not the case, the promoters can be isolated and cloned into appropriate plasmids or vectors by conventional recombinant techniques following the directions provided in Sambrook et al. (*Supra*).

Examples of genetic constructs which have been made are: IL-10 cDNA driven by the insulin promoter (Fig. 10); IL-10 cDNA driven by the immunoglobulin heavy chain promoter (Fig. 11); IL-10 cDNA driven by the IL-6 promoter (Fig. 12) ;IL-10 cDNA under the control of the SRα promoter (Fig. 13) (Takebe, Y. et al. 1988, Mol. Cell Biol. 8:466), and; IL-10 cDNA driven by the IL-2 promoter (Fig. 14).

Similarly, the human TGF-β1 cDNA (Derynck, et al., 1985 <u>Nature</u> 316:8) and other genes for immunosuppressive proteins can be inserted into such vectors. For inducible expression, cDNAS can be cloned directly downstream of either the IL-6 (Libermann, et al., 1990 <u>Mol. Cell. Biol.</u> 10:2327-2334) or the IL-2 (Hoyos, et al., 1989 <u>Science</u> 244:457-460; Fraser, et al., 1991 <u>Science</u> 251:313-316) promoters. Both promoters are highly inducible by several agents, particularly substances released from activated macrophages during immune response (Kishimoto, 1989 <u>Blood</u> 74:1-10; Crabtree, 1982, <u>Science</u> 243:355-361). These inducible promoters have the advantage that IL-10 or TGF-β will be provided by the T cells primarily during acute immune responses,

thus potentially limiting possible adverse effects of IL-10 or TGF-$\beta$.

The different expression vector constructs can be introduced into alloreactive T cells by standard transfection methods. For stable transfection of T cells, electroporation is one useful method (Takahashi, et al., 1991, Exp. Hematol. 19:343-346). IL-10 and TGF-$\beta$ constructs can be transfected together for a possible synergism between IL-10 and TGF-$\beta$ in prevention of allograft rejection.

To select for stably transfected T cells, 1/20 of the amount of DNA of the RSV-neo vector which contains the neomycin gene under the transcriptional control of the RSV LTR (Ausubel, et al., 1991 Current Protocols in Molecular Biology) can be co-transfected together with the IL-10 or TGF-$\beta$ constructs. Transfected cells can be selected by growing in geneticin 418-containing medium.

To evaluate expression of IL-10 or TGF-$\beta$ by the transfected cells, several different assays, such as the IL-10 ELISA assay, described in Current Protocols in Immunology (Mosmann, 1991 In: Current Protocols in Immunology; 1:6.14.1-6.14.8) herein incorporated by reference, can be used. IL-10 activity is determined by the ability of IL-10 to inhibit cytokine production by activated macrophages; this can be measured in a bioassay (Fiorentino, D.F. 1991, J. Immunol. 147: 3815) herein incorporated by reference. TGF-$\beta$ expression can be measured by either the thymocyte proliferation assay in the presence or absence of neutralizing anti-TGF-$\beta$1 antibodies (Genzyme Corporation, Cambridge, MA) or by a radioreceptor assay using A549 lung carcinoma cells (ATCC) as described (Mosmann, 1991 In: Current Protocols in Immunology; 1:6.14.1-6.14.8).

Activation of a pro-drug

A gene encoding an enzyme that activates an immunosuppressive drug can be cloned and expressed in T-cells or in a graft. Expression of a gene such as cyclosporine synthetase (Lawen, A. 1990, J. Biol. Chem. 265: 11355) by the graft would activate the drug, e.g., a cyclsporine precursor, and create an environment of local immunosuppression, thus allowing improved survival of the grafted tissue or organ.

Another immunosuppressive agent that must be metabolized before becoming active is cyclophosphamide. Cyclophosphamide metabolism normally takes place in liver microsomes. The genes encoding the activating enzymes may be cloned and expressed in T cells or engrafted organs, resulting in local activation of the drug.

<u>Enzymes that act to produce a compound capable of inhibiting rejection of a tissue or organ.</u>

Molecules that are the target for preformed antibodies, which present the biggest barrier for xenotransplants, are largely molecules that are heavily glycosylated (Geller et al, 1992, Transplant. Proc. 24:592). The glycosylation sites appear to be targets for these antibodies. Antigenic determinants are frequently located on N-linked substitutions. To eliminate the targets for such xenoreactive natural antibodies, a glycosidase can be expressed in the transplanted organ.

<u>Introduction of Recombinant Genes into Cells of Tissues</u>

A number of methods can be employed for the introduction of recombinant genes into cells of a tissue or organ.

<u>A. Transfection of Dispersed Cells from a Tissue or Organ</u>

Transfection of dispersed cells from a tissue/organ can be accomplished by any number of known methods, including calcium phosphate precipitation, DEAE

dextran, electroporation, and liposome mediated transfection. The method to be used for any given population of cells will depend upon the cell type that is being transfected and their sensitivity to the components of the transfection mixture. Each of these methods is well known to those skilled in the art, and are described in detail in Sambrook et al. (*Supra*).

B. Transfection of Whole Tissues or Organs

Tissue or organs, such as pancreatic islets, can be transfected using either electroporation (BioRad) as described by Welsh et al (Welsh, et al., 1990 Proc. Natl. Acad. Sci USA 87:5807-5811), lipofectin reagent (GIBCO-BRL), pH-sensitive liposomes as described by Welsh et al. (Welsh, et al., 1990 Biomed. Biochim. Acta 49:1157-1164) or microparticle bombardment with the biolistic PDS-1000/He system (BioRad) (Yang, et al., 1990 Proc. Natl. Acad. Sci USA 87:9568-9572).

Transgenic Organs. Xenotransplantation of organs across species barriers has been proposed as a source of donor organs (Auchincloss, 1990 Transplant Rev. 4:14). In particular, experiments with pigs as xenograft donors for human patients are in progress (Gustafsson, et al., 1990 J. Immunol. 145:1946-1951). To prevent xenograft rejection, IL-10, TGF-$\beta$, or another protein with immunosuppressive activity can be expressed in the transgenic organ in either a tissue-specific or inducer-specific manner. In order to express IL-10 and/or TGF-$\beta$ in either pancreatic islets or T cells of transgenic animals, the expression vector constructs described above can be used. For example, the HTLV I LTR driven IL-10 and TGF-$\beta$ constructs can be used for T cell-specific expression, and the insulin promoter-driven IL-10 and TGF-$\beta$ constructs can be used for pancreatic islet cell-specific expression.

For production of transgenic animals, expression vector constructs will be linearized and injected into oocytes according to standard protocols (Sarvetnick, et al., 1988 <u>Cell</u> 52:773-782). Expression of the transgenes can be evaluated by sacrificing a second generation animal and isolating mRNA from different organs and from multiple hematopoietic cell types. Since constitutive expression of such genes in transgenic animals might lead to severe developmental defects or other pathological changes, the transgenic animals preferably contain genes of interest under the transcriptional control of inducible promoters as described above, such as the IL-6 promoter. IL-6 promoter driven constructs should be inducible in a variety of different cell types including endothelial cells, T cells, macrophages, fibroblasts, stromal cells, and mesangial cells (Kishimoto, 1989 <u>Blood</u> 74:1-10).

A nucleic acid encoding the suppressor factor of the invention can be used therapeutically to alter the effects of IL-2 on IL-2R bearing cells, or of IL-4 on IL-4R bearing cells. Four examples of how this method could be used include, but are not limited to, the following. 1) Cells that are targets of autoreactive IL-2R- or IL-4R- bearing cells can be transfected with a nucleic acid encoding the suppressor factor. The cell expresses and secretes the suppressor factor, which causes an alteration of the IL-2 effect, such that IL-2 dependent proliferation of autoreactive T-cells proximal to the cell are subject to inhibition by the suppressor factor. 2) Cells that are in close proximity to cells, e.g., autoreactive T-cells, as they circulate, such as the endothelial cells lining the blood vessels, can be transfected with nucleic acid encoding the suppressor factor. The suppressor factor protein would be released into the blood stream wher it alters the effect of IL-2

on circulating IL-2R bearing cells. 3) In a similar manner, epithelial cells transfected with a nucleic acid of the invention may secrete suppressor factor protein, altering the effect of IL-2 on proximal IL-2R bearing cells. Epithelial cells, e.g., epithelial cells of the kidney proximal tubule and of the gut, activate or inactivate T-cell proliferation and are thus expected to come into proximity with them. 4) Finally, autoreactive T-cells may themselves be transfected with the nucleic acid of the invention, such that the T-cells make and secrete suppressor factor which would in turn alter the effect of IL-2 on themselves. Cells transfected in the manner of the methods above are expected to equally alter the effect of IL-4 on IL-4R bearing cells.

Cells to be transfected can be accessed in several ways, either by removing the cells from a patient for transfection, or by administering a vector, e.g., a virus, comprising a suppressor factor-encoding nucleic acid to the patient. Alternatively, cells, e.g., cells of a cell line, or of a graft, can be transfected *in vitro* and then introduced to the patient.

TABLE I:



MURINE OLIGONUCLEOTIDE PRIMER SEQUENCES

$T_b1$

| IL-2 (MOL BIOL REP 11:57) SIZE | | | PRODUCT |
|---|---|---|---|
| SEQ ID NO: 13 | 5' sense | CTTGGCATGCTTGTCAACAGCGCACCCACT (87-115) | 494bp |
| SEQ ID NO: 14 | 3' antisense | GTGTTGTAAGCAGGAGGTACATAGTTA (555-581) | |
| IFN$\gamma$ (PNAS 80:5842) | | | |
| SEQ ID NO: 15 | 5' sense | CACGGCACAGTCATTGAAAGCC (136-157) | 365bp |
| SEQ ID NO: 16 | 3' antisense | TTCCGGCAACAGCTGGTGGACC (501-480) | |
| $T_b2$ | | | |
| IL-4 (Nucleic Acid Res 15:333. PNAS 87:857) | | | |
| SEQ ID NO: 17 | 5' sense | GATGTGCCAAACGTCCTCACAGC (215-237) | 182bp |
| SEQ ID NO: 18 | 3' antisense | CGATGAATCCAGGCATCGAAAAGC (397-374) | |
| IL-10 (Science 248:1230) | | | |
| SEQ ID NO: 19 | 5' sense | CTGCCTGCTCTTACTGACTGGC (98-120) | 413bp |
| SEQ ID NO: 20 | 3' antisense | AATCACTCTTCACCTGCTCC (511-491) | |
| CTL | | | |
| Granzyme B (Science 232:858) | | | |
| SEQ ID NO: 21 | 5' sense | CCCAGGCGCAATGTCAAT (403-420) | 330bp |
| SEQ ID NO: 22 | 3' antisense | CCAGGATAAGAAACTCGA (732-715) | |
| OTHER | | | |
| $TGF_b$ (J. Immunology 141:690) | | | |
| SEQ ID NO: 23 | 5' sense | AAGTGGATCCACGAGCCCAA (1277-1298) | 244bp |
| SEQ ID NO: 24 | 3' antisense | CTGCACTTGCAGGAGCGCAC (1521-1502) | |
| IL-2R$\alpha$ (JI 134:4212) | | | |
| SEQ ID NO: 25 | 5' sense | GGTCTATATGCGTTGCTTAGG (275-295) | 430bp |
| SEQ ID NO: 26 | 3' antisense | CTCGGGAGAAGAATTTCTGC (705-686) | |
| CONTROLS | | | |
| $\beta$-actin (J MOL Evol 23:11) | | | |
| SEQ ID NO: 27 | 5' sense | CGTGACATCAAAGAGAAGCTGTGC (630-654) | 369bp |
| SEQ ID NO: 28 | 3' antisense | GCTCAGGAGGAGCAATGATCTTGAT (1000-979) | |
| PREPROINSULIN II (J. Mol Evol 23:305) | | | |
| SEQ ID NO: 29 | 5' sense | ACCAGCCCTAAGTGATCCGC (955-975) | 369bp |
| SEQ ID NO: 30 | 3' antisense | GGTAGAGGGAGCAGATGCTGGTGC (1931-1907) | |
| TCR C$\beta$ (Nature 316:828) | | | |
| SEQ ID NO: 31 | 5' sense | GACCCTCAGGCCTACAAGGAGAGC | 267bp |
| SEQ ID NO: 32 | 3' antisense | GGATCTCATAGAGGATGGTKGCAG | |

TABLE 2: Transcriptional Activity on Post-transplant Day 8

| | GRAFT | | | | SPLEEN | | | |
|---|---|---|---|---|---|---|---|---|
| | IL-2 | IFNT | IL-4 | GRAN B | IL-2 | IFNT | IL-4 | GRAN B |
| NO TREAT | 5/5* | 5/5 | 1/5 | 5/5 | 5/5 | 5/5 | 1/5 | 5/5 |
| $DAB_{486}$ IL2 | 0/4 | 0/4 | 2/4 | 3/4 | 0/4 | 0/4 | 2/4 | 3/4 |
| AntiCD3 | 0/4 | 0/4 | 4/4 | 3/4 | 0/4 | 0/4 | 3/4 | 4/4 |

* The numerator = the number of grafts bearing a given mRNA. The denominator = the number of grafts analyzed.

TABLE 2

TABLE 3: IS-2.15 clone proliferates to syngeneic spleen cells and pancreatic islets[a].

| | | NOD cells | |
|---|---|---|---|
| Clone | Media alone[b] | islets[c] | spleen[d] |
| IS-2.15 | 574±47 | 1,727±61 | 1,529±144 |
| #3 | 625±57 | 543±26 | 1,414±115 |
| #5 | 801±37 | 1,066±184 | 1,487±169 |

[a] Results are expressed as cpm ± 1SEM.

[b] complete RPMI.

[c] 5-7 islets/well

[d] 1 x $10^5$ cells/well

TABLE 3

TABLE 4: Cytokine gene expression by PCR of islet-infiltrating NOD T-cell clones[a].

| | NOD T-cell clones | | | | | |
|---|---|---|---|---|---|---|
| | IS-2.15 | | H-4 | | G-3 | |
| Stimulation[b] | NS | S | NS | S | NS | S |
| IL-2 | - | - | - | - | - | - |
| IL-4 | - | - | + | + | - | - |
| IL-6 | + | + | + | + | + | + |
| TNF-α | + | + | - | + | - | + |
| IFN -γ | + | + | + | + | + | + |
| TGF -β | + | + | - | - | - | - |

[a] RNA was extracted from $10^6$ cells by NP-40 method and reverse transcribed using random hexamer oligo$(dN)_6$ as primer in a 50 µl reaction. Amplification reaction was performed using primers specific for sequences of each of the listed genes for 45 cycles.

[b] NS: non stimulated; S: stimulated. T-cell clones activation was achieved by culturing these cells for 4 days with irradiated syngeneic spleen cells.

TABLE 4

Claims

1. A substantially pure protein characterized in that

it is secreted by cloned anergic T-cells,

it blocks IL-2 stimulated T-cell proliferation,

it has an apparent molecular weight of between 10 and 30 kilodaltons,

it can be inactivated by heating to 65°C for 15 minutes,

it blocks IL-4 stimulated T-cell proliferation *in vitro*,

it is non-cytotoxic to T-cells, and

it does not inhibit the production of IL-2 by T-cells *in vitro*.

2. A purified nucleic acid encoding the protein of claim 1.

3. A method of altering the effect of IL-2 on an IL-2 receptor-bearing cell in a mammal, said method comprising

bringing into close proximity with said cell a second cell of said mammal which is transfected with the nucleic acid of claim 2 so that said second cell secretes said protein.

4. The method of claim 3, wherein said second cell is a T-cell.

5. The method of claim 3, wherein said second cell is an endothelial cell lining a blood vessel.

6. The method of claim 3, wherein said second cell is an epithelial cell.

7. The method of claim 6, wherein said epithelial cell is of the proximal tubule of the kidney.

8. The method of claim 7, wherein said epithelial cell is a gut epithelial cell.

9. The method of claim 3, wherein said mammal is a human.

10. A method of altering the effect of IL-2 on an IL-2 receptor-bearing cell in a mammal, comprising,

transfecting said cell with the nucleic acid of claim 2 so that said cell secretes said protein.

11. A method of altering the effect of IL-4 on an IL-4 receptor-bearing cell in a mammal, said method comprising

bringing into close proximity with said cell a second cell of said mammal which is transfected with the nucleic acid of claim 2 so that said second cell secretes said protein.

12. The method of claim 11, wherein said second cell is a T-cell.

13. The method of claim 11, wherein said second cell is an endothelial cell lining a blood vessel.

14. The method of claim 11, wherein said second cell is an epithelial cell.

15. The method of claim 14, wherein said epithelial cell is of the proximal tubule of the kidney.

16. The method of claim 14, wherein said epithelial cell is a gut epithelial cell.

17. The method of claim 11, wherein said mammal is a human.

18. A method of altering the effect of IL-4 on an IL-4 receptor-bearing cell in a mammal, said method comprising

transfecting said cell with the nucleic acid of claim 2 so that said cell expresses said protein.

19. A human T-cell clone characterized in that it

is anergic;

is dependent on recombinant human IL-2 for growth;

expresses cell surface CD8;

is non-cytolytic; and,

expresses Vβ11 T cell receptor.

20. A method of inhibiting rejection of a transplanted tissue in a mammal, said method comprising

introducing into a cell of said tissue DNA encoding an immunosuppressive protein and

transplanting said tissue into said mammal.

21. The method of claim 20 wherein said tissue is an allograft.

22. The method of claim 20 wherein said tissue is a xenograft.

23. The method of claim 20 wherein said DNA encodes IL-10.

24. The method of claim 20 wherein said DNA encodes TGF-$\beta$.

25. The method of claim 20 wherein expression of said DNA is constitutive.

26. The method of claim 20 wherein expression of said DNA is inducible by a compound that stimulates an immune response.

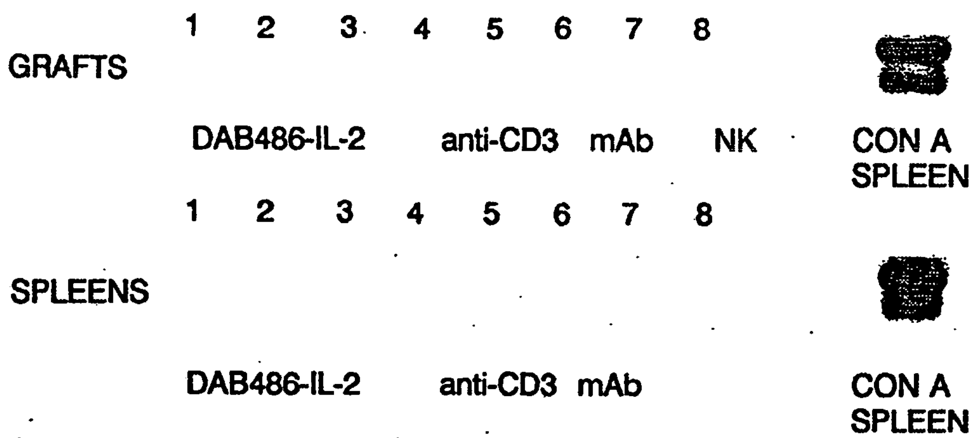
1 2 3 4 5 6 7 8
GRAFTS
DAB486-IL-2 anti-CD3 mAb NK
CON A SPLEEN
1 2 3 4 5 6 7 8
SPLEENS
DAB486-IL-2 anti-CD3 mAb
CON A SPLEEN

FIG. 1

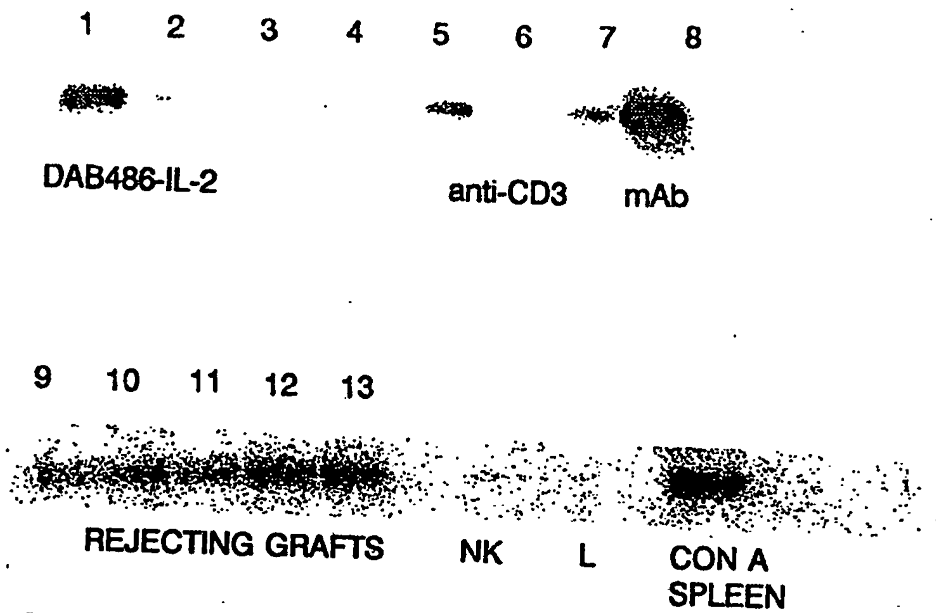
1
2
3
4
5
6
7
8
DAB486-IL-2
anti-CD3 mAb
9
10
11
12
13
REJECTING GRAFTS
NK
L
CON A SPLEEN

FIG. 2

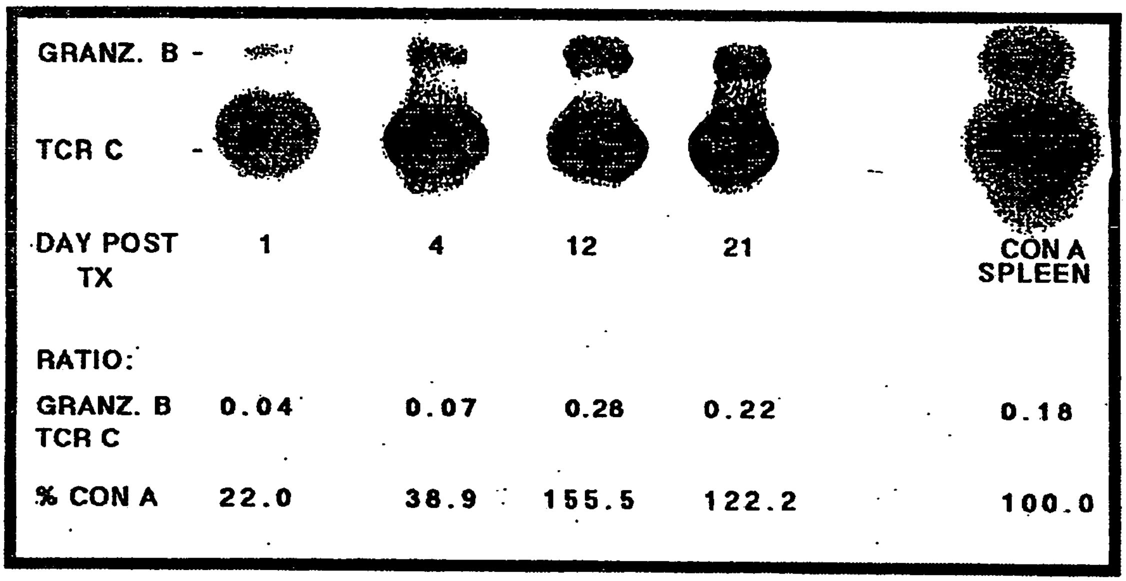
GRANZ. B -
TCR C -
DAY POST TX
1
4
12
21
CON A SPLEEN
RATIO:
GRANZ. B / TCR C
0.04
0.07
0.28
0.22
0.18
% CON A
22.0
38.9
155.5
122.2
100.0

FIG. 3

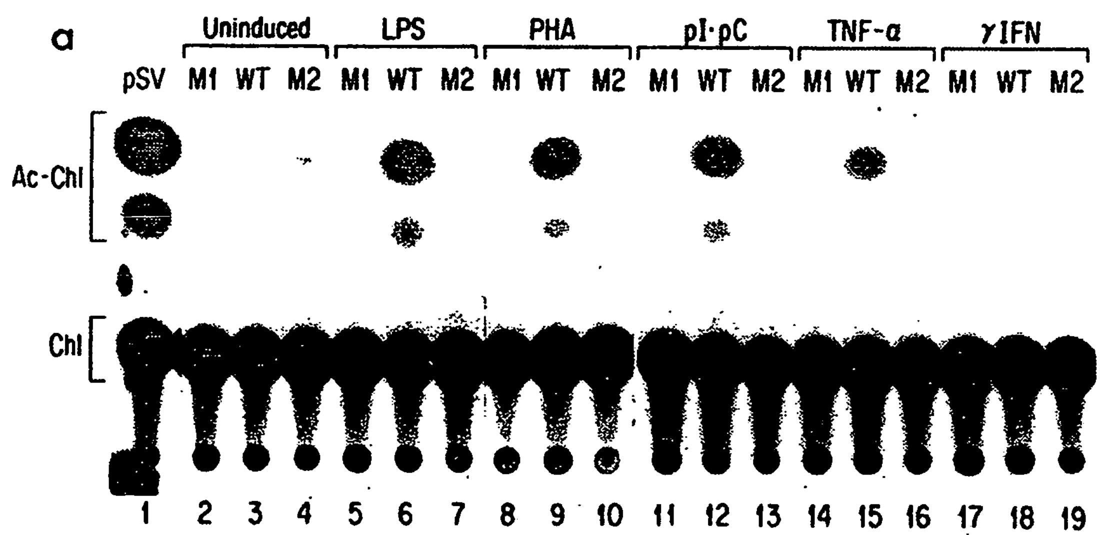
a
Uninduced
LPS
PHA
pI·pC
TNF-α
γIFN
pSV
M1 WT M2 M1 WT M2 M1 WT M2 M1 WT M2 M1 WT M2 M1 WT M2
Ac-Chl
Chl
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

FIG. 4A

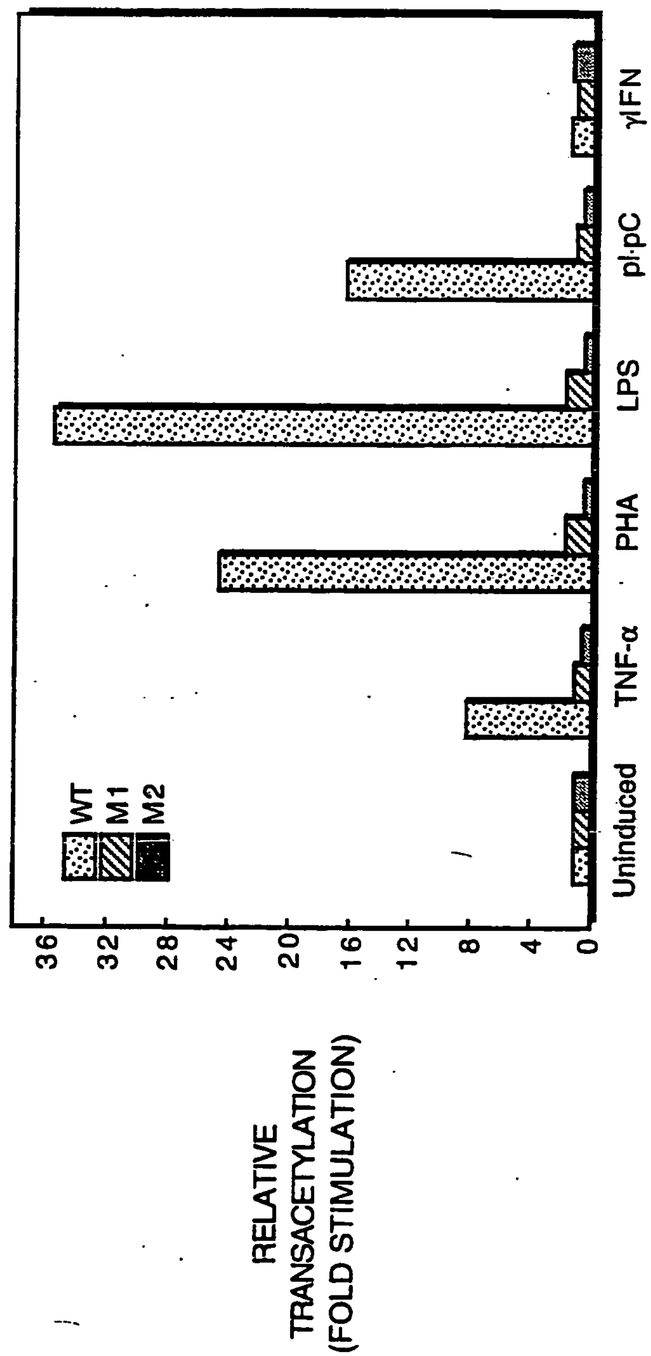
RELATIVE TRANSACETYLATION (FOLD STIMULATION)
0
4
8
12
16
20
24
28
32
36
WT
M1
M2
Uninduced
TNF-α
PHA
LPS
pI:pC
γIFN

FIG. 4B

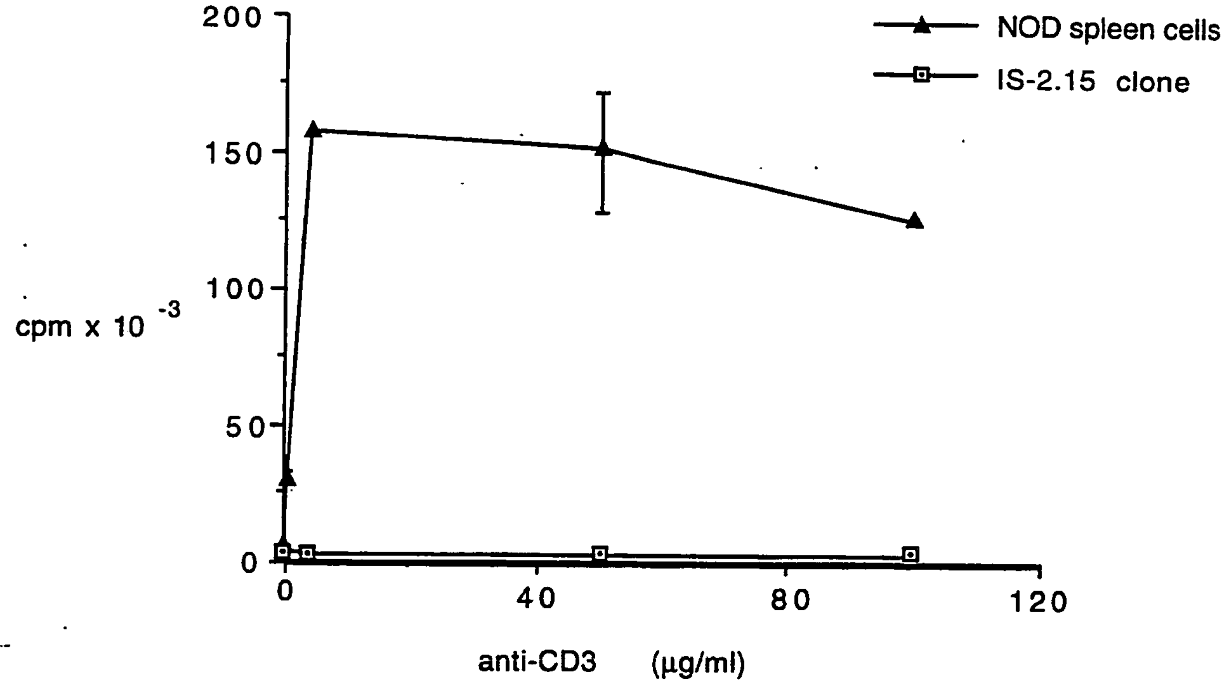
200
150
100
50
0
cpm x 10 -3
0
40
80
120
anti-CD3 (µg/ml)
NOD spleen cells
IS-2.15 clone

FIG. 5A

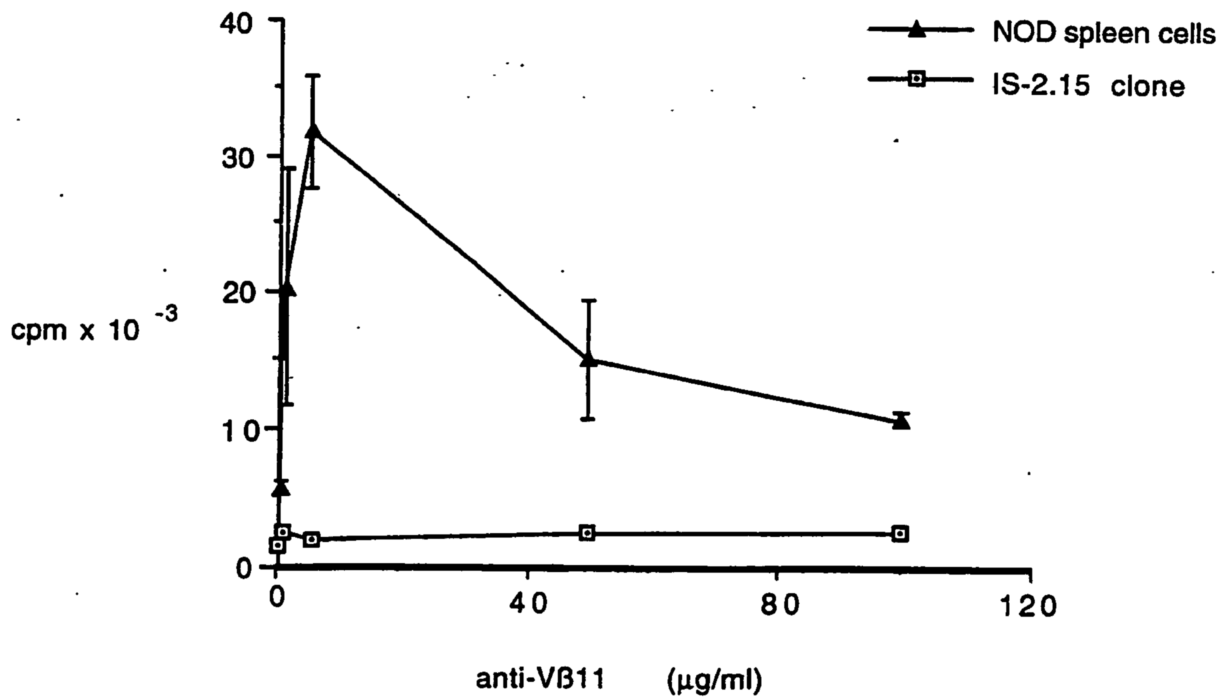
40
30
20
10
0
cpm x 10 -3
0
40
80
120
anti-Vß11 (µg/ml)
NOD spleen cells
IS-2.15 clone

FIG. 5B

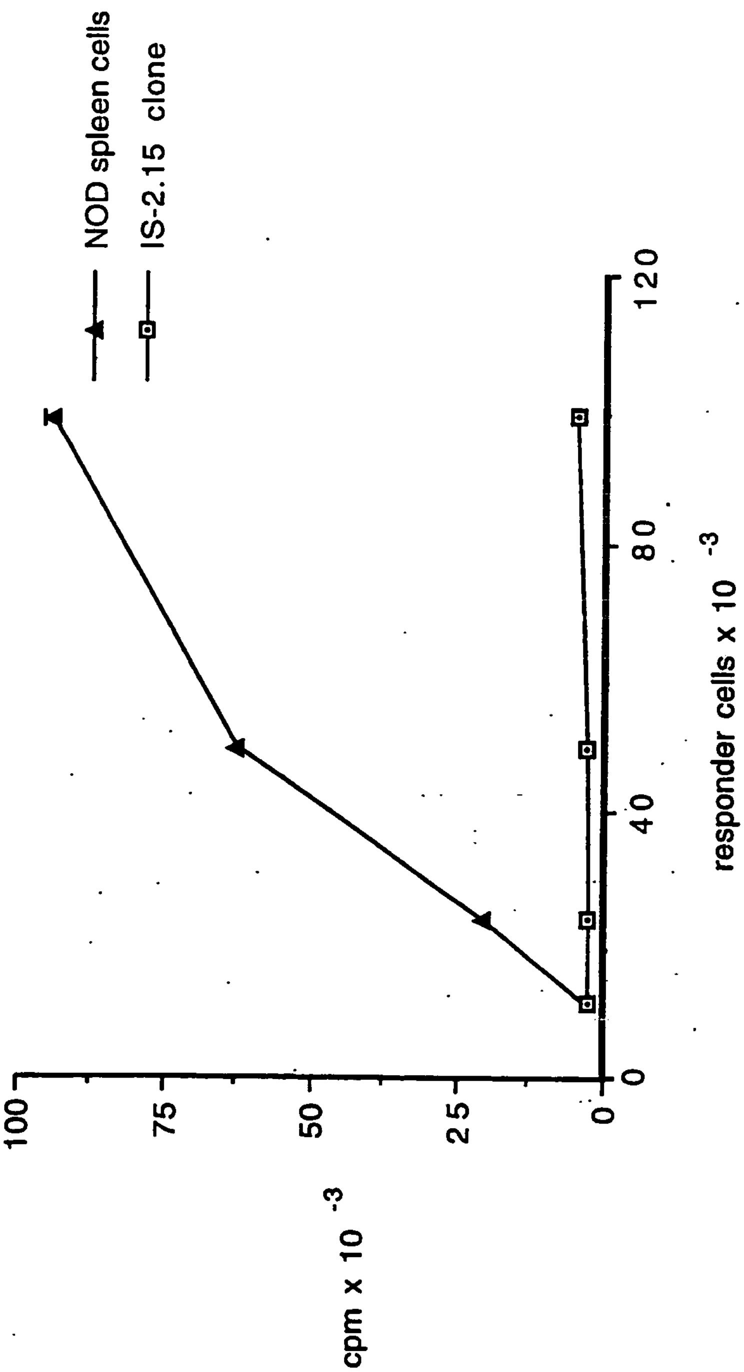
NOD spleen cells
IS-2.15 clone
100
75
50
25
0
cpm x 10 -3
0
40
80
120
responder cells x 10 -3

FIG. 5C

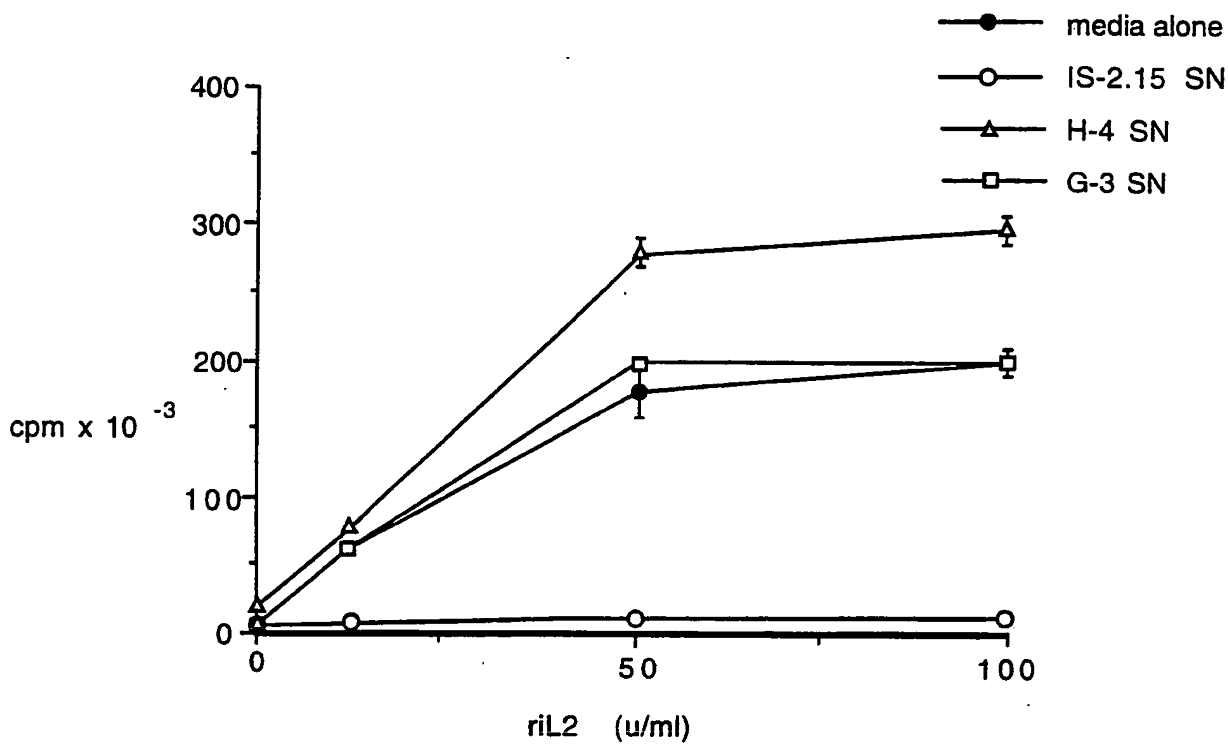
media alone
IS-2.15 SN
H-4 SN
G-3 SN
400
300
200
100
0
cpm x 10 -3
0
50
100
riL2 (u/ml)

FIG. 6A

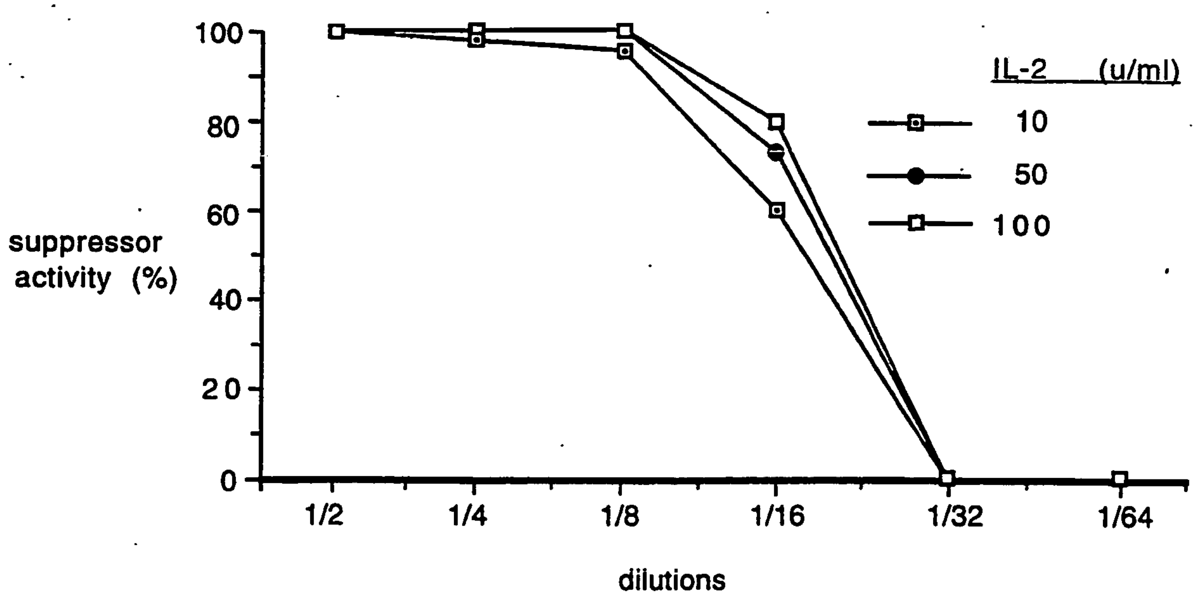
100
80
60
40
20
0
suppressor activity (%)
IL-2 (u/ml)
10
50
100
1/2
1/4
1/8
1/16
1/32
1/64
dilutions

FIG. 6B

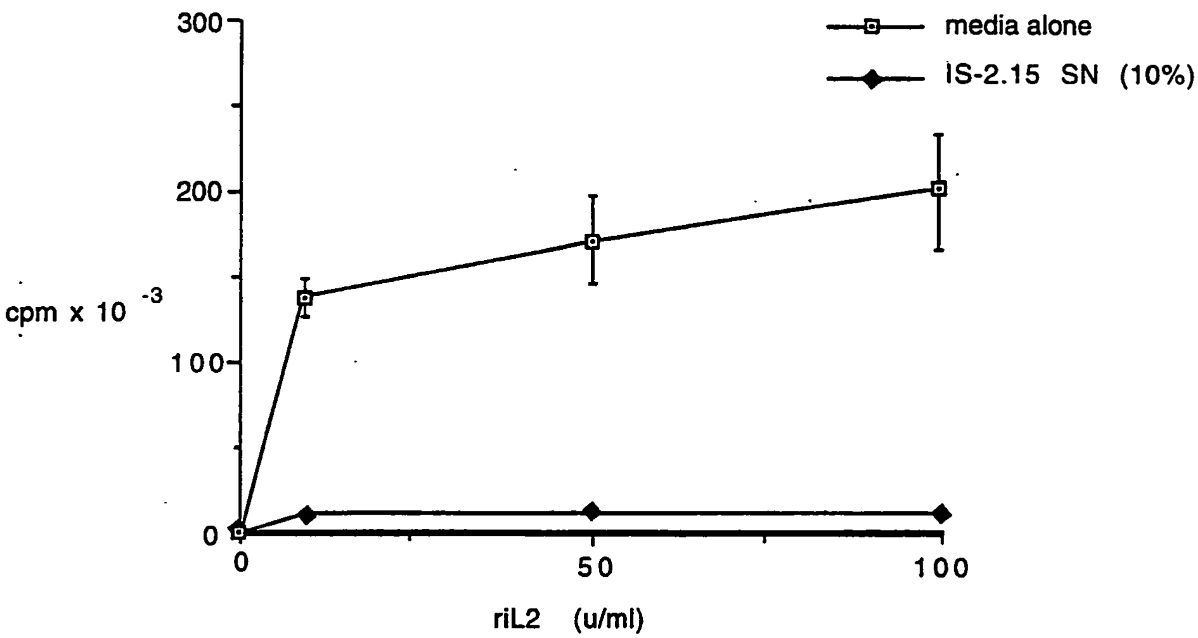
300
200
100
0
cpm x 10 -3
0
50
100
riL2 (u/ml)
media alone
IS-2.15 SN (10%)

FIG. 7

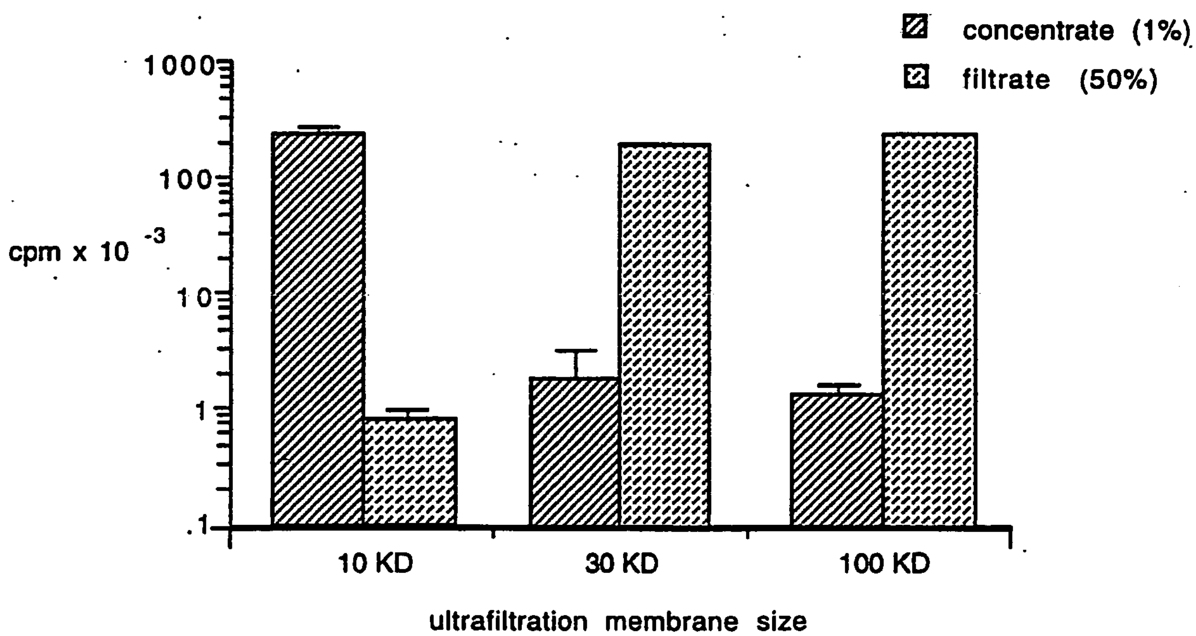
concentrate (1%)
filtrate (50%)
1000
100
10
1
.1
cpm x 10 -3
10 KD
30 KD
100 KD
ultrafiltration membrane size

FIG. 9

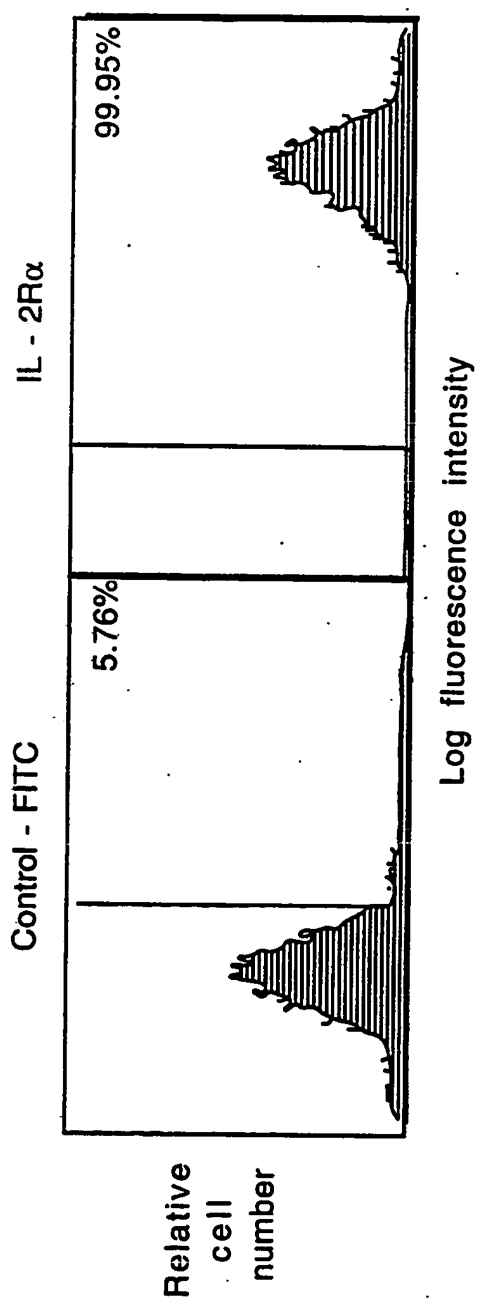
Control - FITC
IL - 2Rα
5.76%
99.95%
Relative cell number
Log fluorescence intensity

FIG. 8A

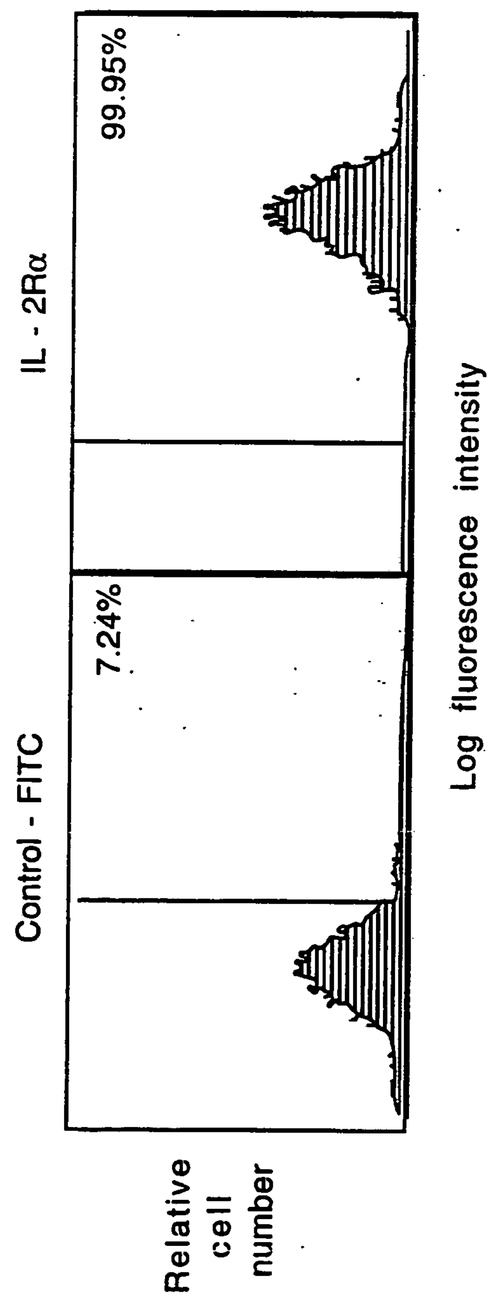
Control - FITC
IL - 2Rα
7.24%
99.95%
Relative cell number
Log fluorescence intensity

FIG. 8B

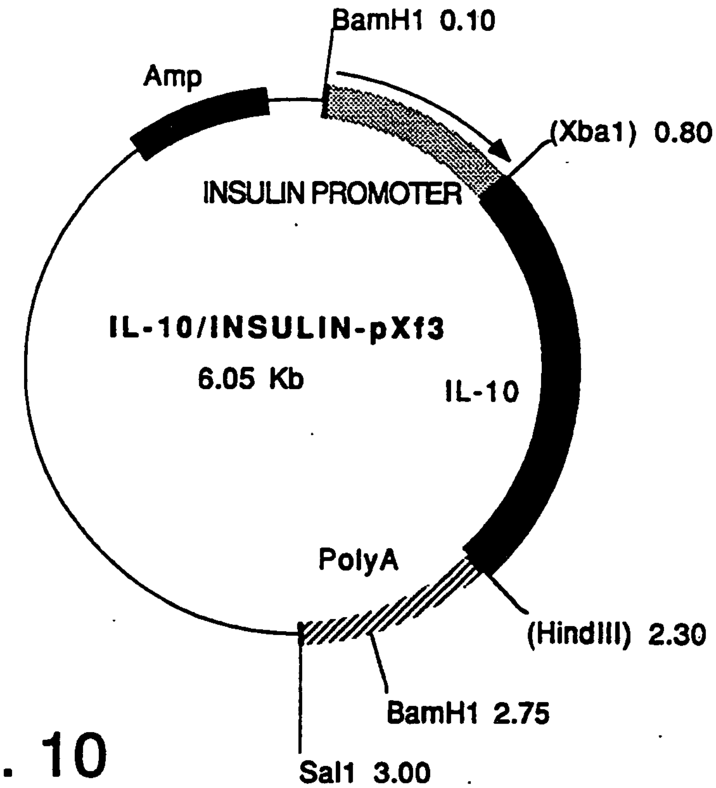
BamH1 0.10
Amp
(Xba1) 0.80
INSULIN PROMOTER
IL-10/INSULIN-pXf3
6.05 Kb
IL-10
PolyA
(HindIII) 2.30
BamH1 2.75
Sal1 3.00

FIG. 10

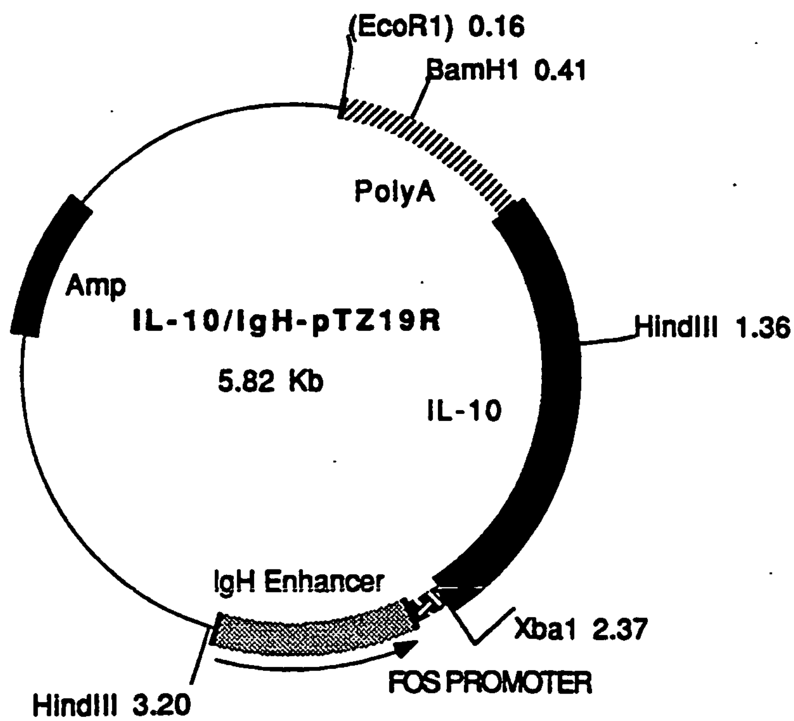
(EcoR1) 0.16
BamH1 0.41
PolyA
Amp
IL-10/IgH-pTZ19R
5.82 Kb
HindIII 1.36
IL-10
IgH Enhancer
Xba1 2.37
FOS PROMOTER
HindIII 3.20

FIG. 11

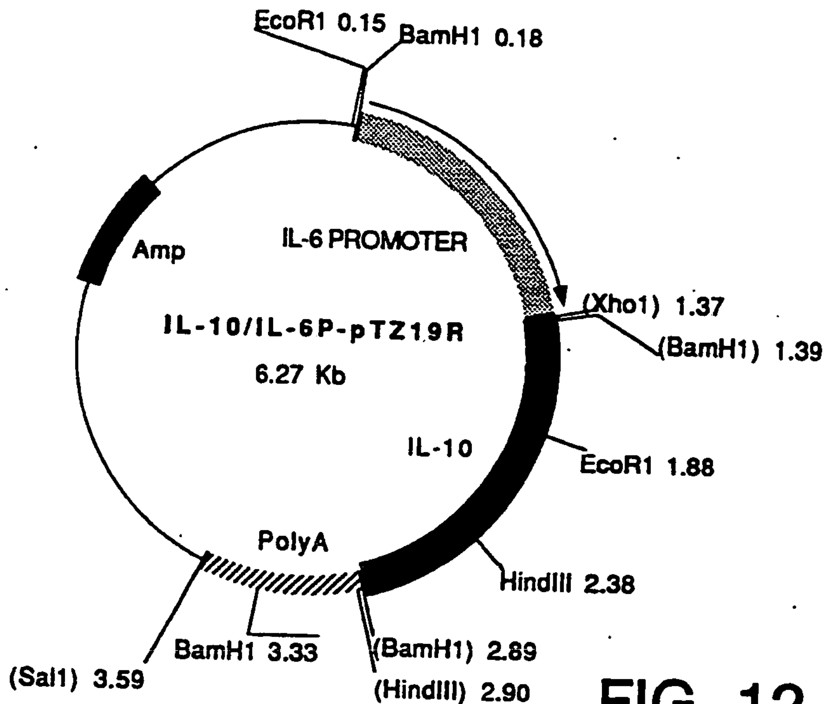
EcoR1 0.15
BamH1 0.18
IL-6 PROMOTER
Amp
(Xho1) 1.37
(BamH1) 1.39
IL-10/IL-6P-pTZ19R
6.27 Kb
IL-10
EcoR1 1.88
PolyA
HindIII 2.38
(BamH1) 2.89
(HindIII) 2.90
BamH1 3.33
(Sal1) 3.59

FIG. 12

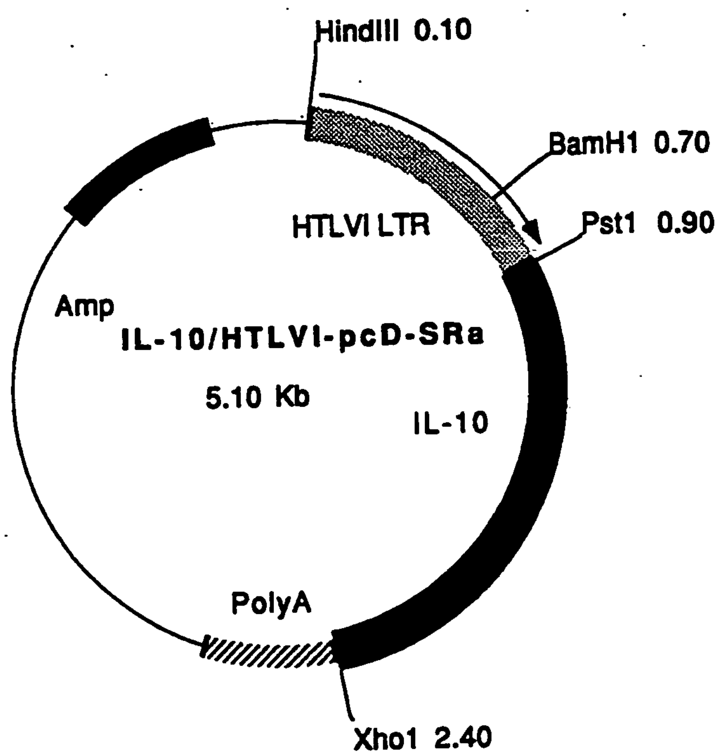
HindIII 0.10
BamH1 0.70
HTLVI LTR
Pst1 0.90
Amp
IL-10/HTLVI-pcD-SRa
5.10 Kb
IL-10
PolyA
Xho1 2.40

FIG. 13

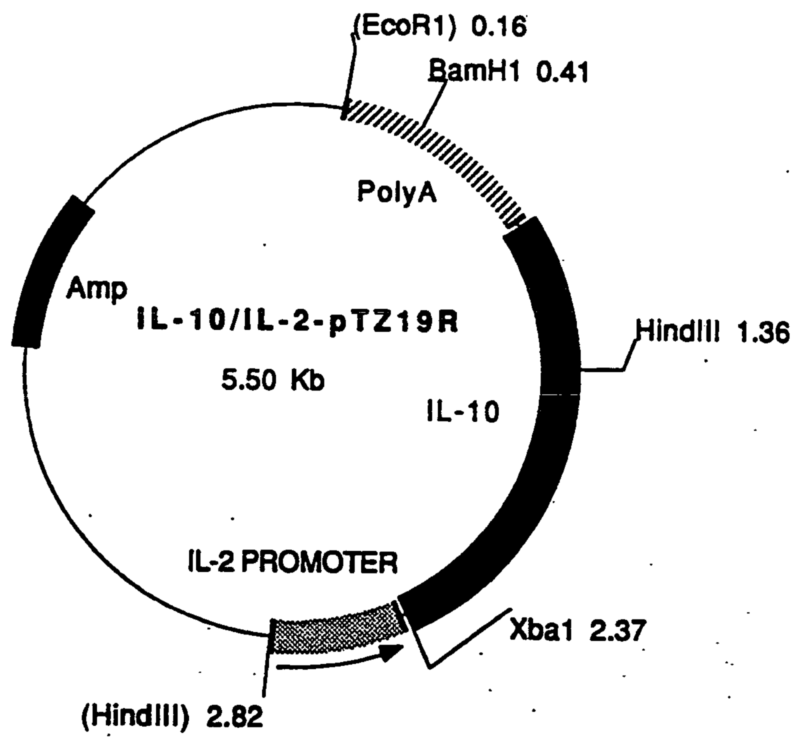
(EcoR1) 0.16
BamH1 0.41
PolyA
Amp
IL-10/IL-2-pTZ19R
5.50 Kb
HindIII 1.36
IL-10
IL-2 PROMOTER
Xba1 2.37
(HindIII) 2.82

FIG. 14

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/01768

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(5) :C07K 15/00; C07H 15/12; A61K 48/00
US CL :530/350, 351, 395; 424/85.2, 93R, 93A, 93B; 514/44; 536/23.5
According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350, 351, 395; 424/85.2, 93R, 93A, 93B; 514/44; 536/23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS AND MEDLINE, DIALOG of Suppressor Protein/Factor, or Inhibitory Protein/Factor, or IS-2.15

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | The J. of Immunol., Vol. 134, No. 2, issued February 1985, Schwyzer et al, "Partial Purification and Biochemical Characterization of A T Cell Suppressor Factor Produced by Human Glioblastoma Cells", pages 1003-1009, see all. | 1-10 |
| X | J. Mol. Cell. Immunol., Vol 3, issued 1987, Almawi et al, "Induction of Suppressor by a Murine Nonspecific Suppressor-Induced Cell Line (M1-A5). III. Partial Purification of the Suppressor Cell-Inducing Factors", pages 157-166, see all. | 1-10 |

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance
"E" earlier document published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

| Date f th actual completion f the international search | Date f mailing f th international search report |
|---|---|
| 27 MAY 1993 | 08 JUN 1993 |
| Name and mailing address of the ISA/US<br>Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231 | Authorized fficer<br>GARNETTE D. DRAPER, PRIMARY EXAMINER |
| Facsimile N . NOT APPLICABLE | Telephone No. (703) 308-0196 |

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | The J. Immunol., Vol 146, No 6, issued 15 March 1991, Fidel et al, "Regulation of Granulomatous Inflammation in Murine Schistosomiasis", pages 1941-48, see all. | 1-10 |
| X | Eur. J. Immunol., Vol. 18, issued 1988, Redondo et al, "Inhibition of Interleukin 2-Induced Proliferation of Cloned Murine T cells by Glucocorticoids. Possible involvement of an Inhibitory Protein", pages 1555-1559, see all. | 1-10 |
| X | Int. J. Cancer, Vol., 42, issued 1988, Kuppner et al, "The Glioblastoma-Derived T-cell Suppressor Factor/Transforming Growth Factor Beta2 Inhibits the Generation of Lymphokine-Activated Killer (LAK) Cells", pages 562-567, see all. | 1-10 |
| X,P | US, A, 5,171,841, (Laurence), 15 December 1992, see cols 1-3. | 1-10 |
| X | US, A, 4,675,285, (Clark et al), 23 June 1987, see cols 1-20. | 2 |
| X | Eur. J. Immunol., Vol. 21, issued 1991, Pankewycz et al, "Islet-infiltrating T cell Clones from Non-Obese Diabetic Mice that Promote or Prevent Accelerated Onset Diabetes", pages 873-879, see all. | 1-10 |
| X | Nature, Vol. 345, issued 07 June 1990, Blackman et al, "A Role for Clonal Inactivation in T cell Tolerance of Mls-1", pages 540-42, see all. | 1-10 |
| X | Business Newsweek, issued 28 May 1990, "The Genetic Age", pages 68-83, see all. | 1-10 |

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

Group I, claims 1-10, drawn to a Suppressor Protein, the Nucleic acid (NA) that encode the protein and to methods f using the NA, classified in classes 530, 536, and 424, subclasses 351, 23.5 and 93A respectively.
Group II, claims 11-18, drawn to methods of regulating IL-4 activity, classified in class 424, subclass 93A
Group III, claim 19, drawn to a T-cell Clone, classified in class 435, subclass 240.2
Group IV, claims 20-26, drawn to a method of treating transplant rejection, classified in class 514, subclass 44

The claims of these four groups are directed to different inventions which are not linked so as to form a single general inventive concept. Furthermore, the claims in these different groups do not have the same special technical feature in common. For instance, the different therapeutic methods require the use of transformed cells (i.e. Groups I and II); or the NA (i.e. Group IV); and the clone of Groups III is not required for each of the other groups.